

CHARACTERISATION OF A CELL-SURFACE MARKER OF APOPTOSIS

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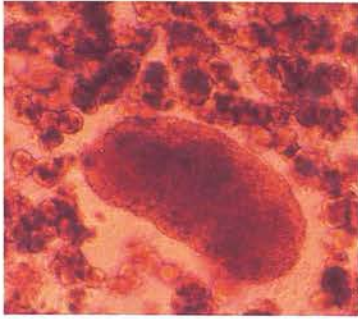


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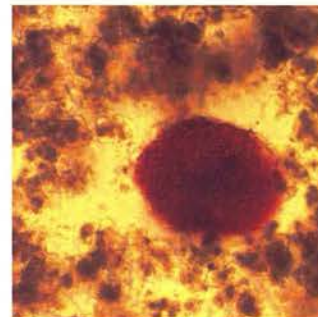
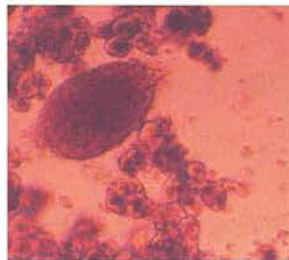
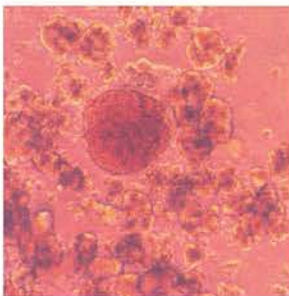
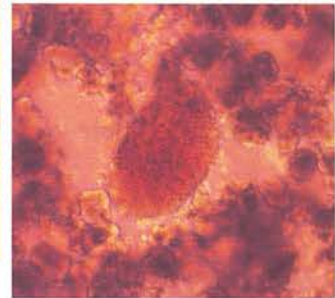
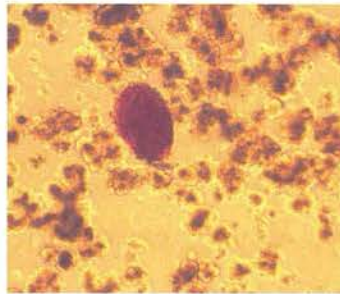
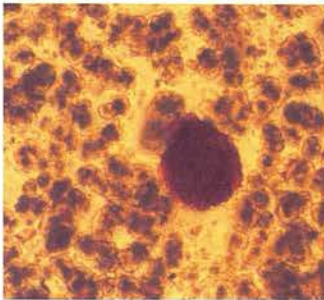
I, Yaw Chong Goh, performed all the experiments presented in this thesis unless otherwise indicated in the text. No part of this work has been, or is being submitted for any other degree of qualification.

Signature:

Date: 26 May 2005



Islets of Langerhans



. collagenase at 2.5mg/ml, injected into the common bile duct just below the bifurcation, at a rate of 1.8ml/min, until even the mesenteric pancreatic lobules are well distended; left to digest for 20 minutes, with digestion halted by ice-cold 2mM EDTA; washed in cold HBSS, and separated with low spin at 100g; returned to warm RPMI and stained with dithizone,

ABSTRACT

The studies undertaken in this research project characterised as fully as possible the antigen identified by the monoclonal antibody BOB78. Earlier studies (Hart, Ross et al. 2000) have linked BOB78 to the identification of apoptotic cells. The present project extended this finding by further studies utilising various antibody-based techniques on cancer-derived cell lines in different stages of apoptosis.

The BOB78 antigen was confirmed to be present in normal cells and to be multi-lineage. BOB78 antigen is normally expressed within the cytosol of nucleated cells, but absent in anucleated red blood cells which do not undergo apoptosis. Following depolarisation of mitochondrial transmembrane potential, a key event in the initiation of apoptosis, BOB78 antigen is detected on the surface of the outer cell membrane. This surfacing of the BOB78 antigen parallels that of phosphatidylserine residues, presently the most well characterised marker for apoptosis in analysis with flow cytometry. Immunocytochemistry corroborated this finding by showing the translocation of the BOB78 antigen to membrane blebs of apoptotic cells. Observations using agents which disrupt the endomembrane synthetic pathways suggest that BOB78 is probably not trafficked through the Golgi apparatus or processed for secretion. Although BOB78 is an IgM antibody, it does not identify a carbohydrate epitope and is therefore likely to be specific for a multi-lineage protein.

Membrane blebbing in apoptotic cells appears to share certain characteristics with the budding of platelets from megakaryocytes. For example, the production of platelets during the terminal differentiation of megakaryocytes involves activation of caspases.

Interestingly, BOB78 was found by flow cytometric analysis to be present in platelets as they develop from the membranes of the MEG-01 megakaryocytes. BOB78 was also expressed on the surface of senescent platelets. These MEG-01 platelets were therefore used for purification of BOB78 through immunoprecipitation. The captured BOB78 antigen was sequenced using MALDI-TOF, which identified it as chaperonin, also known as heat shock protein 60 (hsp60).

Heat shock protein 60 is a highly conserved stress protein which has chaperone functions in prokaryotes as well mammalian cells. Expression of hsp60 on the surface of apoptotic cells may serve novel functions or purposes akin to molecular chaperoning. Mechanisms underlying the translocation of hsp60 to the cell membrane have been characterised in various prokaryotes. Homologous pathways of movement for this highly conserved entity are likely to exist in mammalian cells. The observation that hsp60 is expressed on the surface of senescent platelets suggests a possible role as a recognition signal in phagocytosis. Platelets are equipped with caspases and when senescent display membrane changes typical of apoptotic cells. Like apoptotic cells, platelets are normally cleared from tissues by macrophages. Surface expression of hsp60 is probably an essential change marking cellular entities for uptake by phagocytes. It remains to be studied if engulfment of bodies displaying an endogenous moiety like hsp60 might play a key role in mediating a non-inflammatory response observed in apoptosis. It may also be speculated that hsp60 serves the function of molecular mimicry as many intracellular bacteria display surface hsp60, the expression of which has been linked to pathogenic invasiveness. The ability of these microbes to evade immune clearance may be linked to the non-inflammatory apoptotic disguise which surface hsp60 confers on them.

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First and foremost I have to thank National Medical Research Council, Singapore and Professor Soo Khee Chee for their support in this undertaking.

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My wife Celestial brought me on this voyage. She had provided monumental support during bewildering times and trying episodes, and was still able to offer editorial input. My Mother has tolerated my absence. Scotland has always been the only place. As ever the sweetest things just turn up. Thank You!

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Abbreviations

Apaf	apoptosis protease activating factor
CRT	calreticulin
CPT	camptothecin
DISC	death inducing signaling complex
FADD	Fas associated death domain
FLIPs	FADD-like ICE inhibitory proteins
GAS-6	growth arrest specific protein 6
IAP	Inhibitors of apoptosis
ICE	interleukin converting enzyme
MFGF	milk fat globule factor
MTP	mitochondria transmembrane potential
PS	phosphatidylserine
SDS	sodium dodecyl sulphate
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight

CHAPTER 1

INTRODUCTION

The life of a cell may be terminated under a variety of circumstances. Within a multicellular organism the context of cell death may be physiological or pathological. Thus, the loss of a cell may be physiological as it is essential to the restoration of homeostasis; or it may be pathological where a vital equilibrium may be threatened by the death of the cell. Alternatively, cells may be conferred inappropriate longevity, leading to significant perturbations of physiology or the emergence of serious pathologies. Observations on cell death have therefore linked it to many diseases. In a multicellular organism cell death needs to be accurately balanced. The past three decades have witnessed illuminating studies generating a new level of understanding of the events underpinning and regulating cell death. Understanding cell death promises greater insights into developmental processes, degenerative diseases, inflammatory conditions, autoimmune diseases and cancers. Tremendous potential exists for targeted manipulation of cell death and clearance of cells which may translate into significant therapeutic benefits.

Apoptosis: a form of programmed cell death

Clinical specimens featuring overwhelming infections or physico-chemical damage have presented classic examples for pathological cell death. The destruction of cells typically occurs through a process termed necrosis. Necrosis describes the death of cells which occurs when environmental stresses have been overwhelming, too extreme in degree and rapidity, resulting in a direct, contemporaneous and contiguous pattern of

cell loss. The energy-dependent machineries maintaining functional and anatomical compartmentalisation, such as organelles, are destroyed. Membranes lose their selective permeability allowing the ingress of ions and molecules leading to swelling of organelles and cytoplasm. Eventually the cell is lost through uncontrolled physical changes rupturing the cell membrane with haphazard spillage of cellular contents. The microscopic features of necrosis are typical. The tissues involved show a collection of indistinct cellular outlines within which organelles are not discernible and the nuclei usually appear compacted and small.

In contrast to this pathological form of cell death a cell may die through apoptosis. Apoptosis occurs specifically in groups of cells or in a single cell. As implied by its Greek root (falling leaves, as from a tree) apoptosis is not dramatic under the microscope (Kerr, Wyllie & Currie, 1972). Typically, the dying cells are scanty and discrete within the tissues. A single apoptotic cell is usually found amongst normal neighbouring cells. The apoptotic cell itself however shows remarkably consistent changes especially in the nuclei. Since being first described apoptosis has been extensively studied and much is now known about the initiation and completion of the process. The nature of apoptosis which is the striking opposite of necrosis is that the cell mobilises from within the very tools required to deconstruct itself in a controlled and orderly manner. Death through apoptosis is a form of turnover crafted into normal physiology (Wyllie, Kerr & Currie, 1980). It is a form of programmed cell death and is the commonest form of cell death. It is an essential phenomenon in embryogenesis, metamorphosis, endocrine-controlled tissue atrophy, and normal tissue turnover (Meier, Evan et al., 2000; Vaux and Korsmeyer, 1999).

The end-point of apoptotic cell death consists of specific manifestations of cell morphology and biochemistry. The sequence of events leading to apoptosis follows a genetic programme which is highly conserved (Ellis et al., 1991). The many cellular factors enrolled into the act of deconstruction of the cell are therefore conserved throughout evolution. The most enlightening observations have in fact derived from studies of the nematode *Caenorhabditis elegans* (Jacobson, 1997). Many of the genes encoding products which participate in cell deletion during the development of *C. elegans* have been matched to their mammalian homologues. The apoptotic pathways at its initiation, execution and regulatory phases have counterparts matched to various products in the mammalian system. For example CED-4 is an adaptor protein which transduces the signal for initiation of apoptosis and its mammalian homologue is Apaf-1 (Apoptosis protease activating factor-1); CED-3 is a cysteine protease (known as caspase in mammalian cells) which activates downstream substrates leading to typical changes in apoptosis; whilst CED-9 is a regulator, binding to CED-3 to prevent its activation, similar in action to the anti-apoptotic members of the Bcl-2 family of proteins (Ashkenazi and Dixit, 1998; Vaux and Korsmeyer, 1999)

Apoptosis: morphological changes

During apoptosis a cell undergoes dramatic changes in its morphology (Kerr, Wyllie & Currie, 1972). The cell first shrinks and condenses in size, vacuoles then develop in the cytoplasm, nuclear condensation follows; later the nucleus fragments into clumps. The cell then develops evaginations of the plasma membrane described as blebbing, and pockets of cytoplasm get packaged into the membrane blebs. Other membrane changes seen include echinoidal projections (Willingham, 1999). Ultrastructurally the organelles in the cytoplasm maintain their gross integrity whilst the execution phase of apoptosis

proceeds. Compartmentalisation of cytosolic organisation appears to be maintained. Due to the condensed cytoplasm an increased granularity of the apoptotic cell is usually discerned. The chromatin is degraded into neat multiples of internucleosomal slices of around 200 base pairs and the nuclear partition is broken down. The chromosomal materials and the organelles are then trafficked to the plasma membranes, channelled into the blebs and these pinch off from the plasma membrane. The blebbing apoptotic cell presents one of the most dramatic pictures of cell termination and is in stark contrast to the necrotic process. In a word, these changes reflect how a cell committed to apoptosis would lose contact with its neighbouring cells, shut down most cellular maintenance activities such as DNA repair and synthesis, rearrange the organelles, disrupt the nuclear envelope, digest genomic DNA, reorganise the cytoskeleton, and distribute cellular remnants into manageable packages, which would be ready for disposal by other cells.

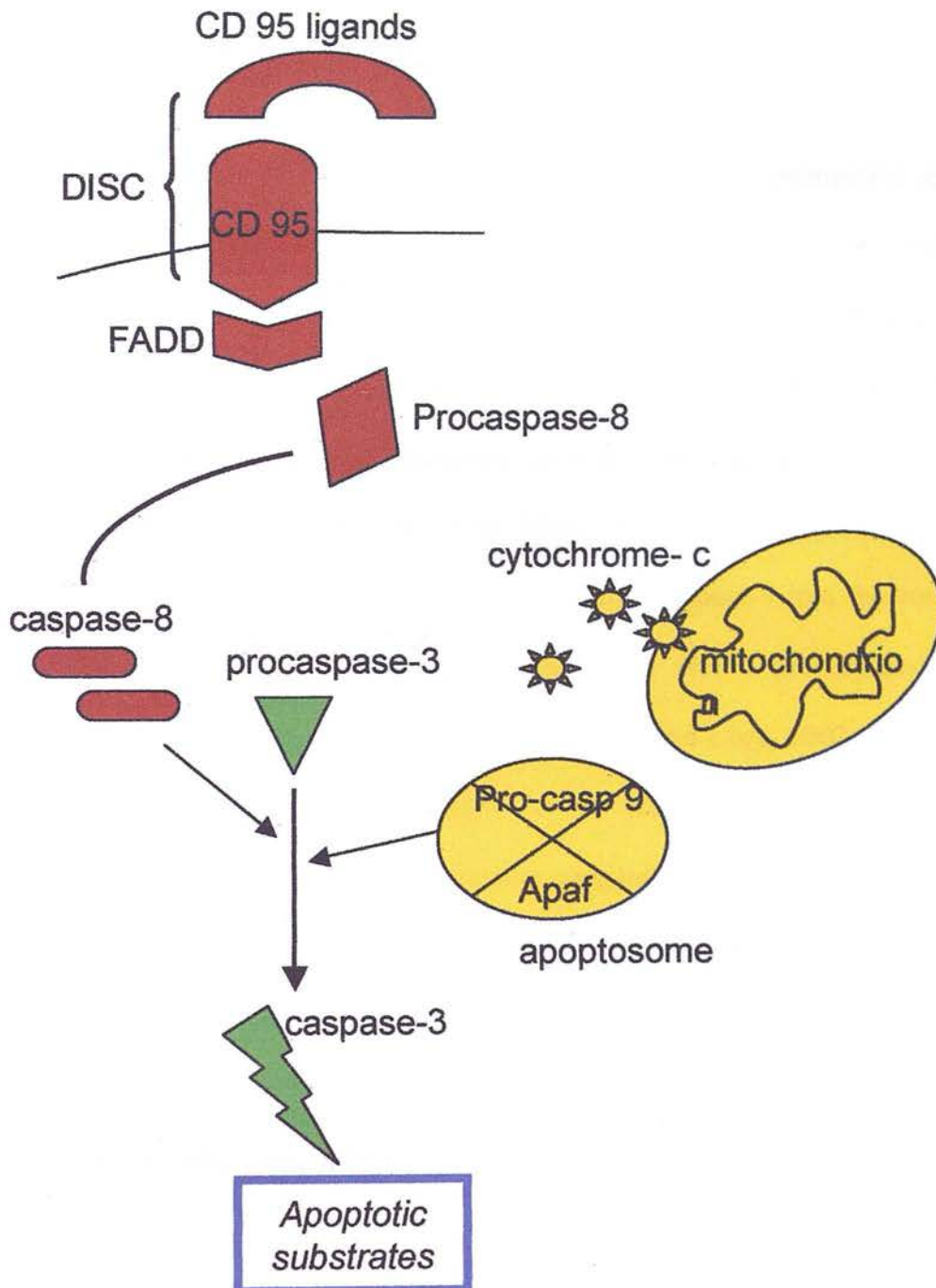
Whereas necrosis is typically attended by an inflammatory reaction to the disorderly array of dying cells and their contents, apoptosis is non-inflammatory (Huynh et al., 2002; Savill et al., 2002). This is an important hallmark of apoptosis. Although the phagocytosis of apoptotic cells may be attended by 'immunological neutrality', as in the failure to stimulate release of pro-inflammatory cytokines; it had been demonstrated that the uptake of apoptotic cells results in active immunosuppressive responses with induced upregulation of anti-inflammatory cytokine eg. TGF- β (transforming growth factor- β) in the phagocytes (Fadok et al., 1998). Apoptosis is particularly important in the immune system (Krammer, 2000). It is the commonest form of cell removal in clonal deletion in the thymus, and in resolution of inflammation (Craxton et al., 1999; Krammer, 1999). Non-phlogistic immune reaction to apoptosis is also pivotal to the

quiet resolution of inflammation and clearance of dead cellular components (Maderna and Godson, 2003). Imbalances in the clearance of immune cells or apoptotic cells play critical roles in the generation of immunological disorders. Appropriate disposal of apoptotic cells avoids inappropriate inflammatory responses and the development of pathology.

Apoptosis: biochemistry of programmed cell death

The primary signal for the termination of a cell may arise from within the cell or the external environment (Hengartner, 2000). These separate pathways can be seen as intrinsic or extrinsic induction of apoptosis (illustrated in figure 1.1 and 1.2). The key initiator in the intrinsic pathway lies with the mitochondria (Loeffler & Kroemer, 2000; Bratton & Cohen, 2001). The transducer in the extrinsic initiation is a membrane molecule called the death receptor (Nagata, 1997). A classic example of death receptor is CD95 also known as Fas/Apo-1, a member of the superfamily of tumour necrosis factor receptors. Both the intrinsic and extrinsic pathways converge on caspase-3, which drives the enzymatic cascade of activation of further caspases instrumental in splitting the cell into membrane-bound parcels. The caspases (cysteinated aspartate specific proteases) are proteolytic enzymes which carry out the cellular disassembly (Thornberry & Lazebnik, 1998; Thornberry, Chapman & Nicholson 2000). To cleave procaspase-3 into its active form, the intrinsic pathway assembles a complex called apoptosome, whilst the extrinsic pathway activates the upstream enzyme caspase-8.

Figure 1.1 Biochemistry of Apoptosis. The *intrinsic* pathway is dependent on the mitochondria whilst the *extrinsic* pathway is initiated by the death receptor.



DISC: Death Inducing Signal Complex;
FADD: Fas-associated Death Domain;
Apaf: Apoptosis protease activating factor

Apoptosis: the intrinsic pathway

When a cell is no longer enduring the stresses placed on it, the energy-generating mitochondria initiates the key event in committing the cell to controlled deconstruction (Hengartner, 2000). Mitochondria lose the electrical potential they maintain between their membranes, the mitochondrial membrane potential (MMP). This potential is maintained through an energy-dependent process. The membrane potential of the mitochondrion is lost whereupon proteins contained in the intermembrane space are released into the cytosol. The prime initiator of apoptosis normally restricted to this space, cytochrome-c, is then released into the cytosol. Other pro-apoptotic factors which are released include caspase-2 and caspase-9. In the cytosol, cytochrome-c interacts with another factor Apaf (apoptosis protease activating factor). Cytochrome-c binds to monomers of Apaf-1 allowing these to assemble into the heptameric apoptosome (Li et al., 1997). Apoptosome recruits procaspase-9 into the complex to cleave and activate it. Apoptosome complexes then cleave procaspase-3, at the convergent point in the initiation of apoptosis (Adams and Cory, 2002). Procaspase-9 requires both cytochrome-c and Apaf-1 for its activation (Cain et al., 1999). Cytochrome-c release is a universal feature in apoptotic cell death (Green and Reed, 1998). In apoptosis which is initiated extrinsically the release of cytochrome-c is a later event and occurs after the caspases have been activated.

Apoptosis: the extrinsic pathway

This system of apoptosis which results from transduction of the death signal through the death receptor also relies on the formation of a complex of molecules (Krammer, 2000); Nagata, 1997). Members of the death receptors family share a similar scheme of substrate activation in the sequence of (1) ligand binding (2) receptor oligomerisation

and formation of DISC (death inducing signal complex) (3) recruitment of the adaptor molecule FADD onto the DISC (4) interaction of DISC with procaspase-8 and (5) cleavage and activation of procaspase-8. This cascade of activation is exemplified by the binding of ligand to Fas/Apo-1 (Baker and Reddy, 1998). This member of the tumour necrosis factor (TNF) receptor family is usually constitutively expressed on cells of the immune system. Its expression can however be induced in other cells as well, especially in inflammatory states. The ligand for CD95/Fas had been identified (Suda & Nagata, 1994). Most members of the death receptor family have corresponding receptors similar to those of TNF-RI and TNF-RII for tumour necrosis factor. Fas-ligand is naturally occurring and may exist as a soluble factor exerting effects distally as well as proximally, or can be expressed on the surface of cells of the immune system, for example, lymphocytes exerting a direct cytotoxic effect upon encounter with a target cell. Upon engagement of the corresponding ligand at the cell-surface Fas oligomerises into a trimer (Ashkenazi and Dixit, 1998). Trimerization is essential for activity and the Fas-trimer engages the FADD (Fas-associated death domain) protein. This interaction happens between the corresponding death domains of Fas and FADD. These then incorporate procaspase-8 into the complex of DISC (death-inducing signalling complex). Procaspase-8 is then cleaved to form the active caspase-8 which is then released into the cytosol, where it will through proteolysis and activate procaspase-3, the convergent point in the initiation of apoptosis. Caspase-8 is the key initiator in the death-receptor pathway. When death-receptors are oligomerised through binding to their ligands, for example, three Fas-ligands to trimerise the Fas/Apo-1, the resulting trimer is thought provide a focal point for several pro-caspase 8 molecules to aggregate. It is predicted that the proximity of the procaspases, with their inherent

catalytic property, might eventually set off enzymatic cleavage of these into active caspases. However the exact activation event of procaspase-8 remains unknown.

Apoptosis is the most common route of disposal for cells of the immune system. The circumstances where the expression of CD95/Fas is most relevant are inflammatory conditions and clonal deletion of cells in the development of immune system. Moreover, as other cells may be induced to upregulate expression of CD95/Fas, such as virus-infected cells, this death-signalling pathway is also the most common form of cytotoxic killing by lymphocytes and natural killer cells.

Executors of apoptosis: caspases

Caspases are the executors of apoptosis (Cryns & Yuan, 1998). They are deployed to bring about the dramatic morphological changes in a cell during apoptosis. Caspases are conserved through evolution and are found in insects, nematodes and hydra (Budihardjo et al., 1999; Cikala et al., 1999; Earnshaw et al., 1999). All enzymes of this family possess an activity specific cysteine, and cleave the Asp-Xxx bonds (i.e. after aspartic acid residues) of the substrates, hence the common name caspases (cysteinated aspartate specific activated proteases). Caspases are involved from the initiation through to the packaging of apoptotic cells. Genes coding for caspases in mammals have been matched to their counterparts in *C elegans*. Caspases act in a cascade where an upstream activated enzyme will, through proteolysis, activate the precursor downstream in a predetermined sequence (Nicholson & Thornberry, 1997). Several properties of caspases appear to be the key to the mobilisation of this cascade upon entry into apoptosis (Thornberry & Lazebnik, 1998). Proteolytic modification of caspases cannot be reversed. This ensures a unidirectional progress along the apoptotic

pathway. However, as expected of a highly regulated process, there are inhibitors of activated caspases at various levels of the cascade (Salvesen & Duckett, 2002). The rapidity with which apoptosis can be effected is linked to a pool of caspases ready for functional mobilisation, as most of the caspases already exist in precursor forms in living cells. The cascade of activation is facilitated by autocatalytic proteolysis caspases have upon themselves. The activation of caspases therefore can proceed exponentially. As cellular disassembly is an ordered sequence of catalytic events, with subcellular compartments undergoing varying degrees of modification, the caspases retain high specificity for the substrates involved. Since most of their actions render shutdown of many of the maintenance machineries in a cell, caspases generally render their substrates inactivated.

Caspases exist in inactive precursor forms in the cell. In this zymogen state they consist of three domains, an N-terminal prodomain, and the p20 (~20kD) and p10 (~10kD) domains. The caspases are cleaved between the domains. The p20 subunit and p10 subunit then associate to form a heterodimer which further associate to form a tetramer with active sites. The active site of each p20/p10 dimer comprises of residues from both subunits and both are essential for substrate binding and catalysis (Walker et al., 1994; Wilson et al., 1994). The active sites uniformly have the residue sequence of Asp-Xxx which enables caspases to effect autocatalytic activation. This positive feedback loop is probably pivotal to the amplification of the cascade. Such sequences of activation group caspases as initiators and effectors.

The caspases are targeted toward specific substrates within the cell. The substrates may either lose their functions or gain new functions. Gain of function results from removal

of regulatory or inhibitory units. In apoptosis most of the caspase substrates are inactivated through proteolysis. Many of these substrates are nuclear related proteins. One of these is a nuclease (known as caspase activated DNase, CAD) which cuts the genomic DNA at internucleosomal segments generating DNA fragments in multiples of roughly 200 base pairs (Nagata et al., 2000). In non-apoptotic cells CAD exists coupled to ICAD in an inactive precursor form. Activation of CAD occurs through caspase-cleavage release of the inhibitory unit it carries (ICAD) (Enari et al., 1998). The DNA repair enzyme PARP (poly(ADP-ribose) polymerase) is disabled through cleavage by the caspases. The changes in the nucleus with attendant shrinkage and clumping are mediated by caspase acting on nuclear lamins (intermediate filament proteins) (Takahashi et al., 1996; Orth et al., 1996). Nuclear lamins which polymerise to form the limiting partition ie. nuclear lamina are cleaved by the caspases, leading to collapse of this structure which contributes to chromatin condensation. The changes in cell shape also result from action of caspases on their substrates (Coleman et al., 2001). These act on the cytoskeleton bringing about cytoplasmic condensation. Caspase-3 acts directly on the cytoskeleton kinases and one of the effect is the phosphorylation of myosin light chain (MLC) which is associated with the formation of blebs from the plasma membrane (Wen et al., 1997; Rudel & Bokoch, 1997).

Caspase-3 is the convergent point of the intrinsic and extrinsic pathways of apoptosis initiation. Occurring as an inactive precursor procaspase-3 it resides in the cytosol within access by the initiators. When cleaved by caspase-8 or the apoptosome as described earlier, the active enzyme catalyses several reactions which initiate the changes in the cytoskeletal structure of the cell and the breakdown of nucleus into oligonucleosomal packets.

Regulatory points in the apoptotic pathways

The patterns of caspase activation involve amplification loops and positive feedbacks which would culminate in a quick death for the cell. Actions of caspases however are subject to regulation. Controls occur at key activation points. Antagonists and agonists for the caspases exist and it may be the integration of the overall effects of these which determine if apoptosis will proceed. This is an interesting area of studies where new factors which inhibit or regulate the caspases are frequently being reported. The following brief review of the better characterised systems will serve to illustrate some known interactions. In the intrinsic pathway at the level of the initiator caspase-9 are the Bcl-2 family of proteins. This is a very large family of proteins the members of which have either anti-apoptotic or pro-apoptotic properties. The various members are involved in balancing apoptotic signals within the cytoplasm, the net of which may depolarise the mitochondrial membrane potential to initiate apoptosis (Adams & Cory, 1998).

Regulators at mitochondria: Bcl-2 family proteins

The proto-oncogene Bcl-2 was first discovered at the chromosomal breakpoint of t(14;18) in human B-cell lymphomas (hence bcl) (Adams & Cory, 1998). To date at least 15 members of this huge family of modulators have been identified. The Bcl-2 proteins comprise of a large family of members which share certain sequence homology. However they have opposing functions and are divided into three groups. Group I members possess anti-apoptotic activity whilst members of groups II and III share pro-apoptotic activity. Prior to activation of apoptosis, the anti-apoptotic members reside on the cytoplasmic side of membranes of mitochondria, endoplasmic reticulum and the nuclear envelope. One of the reasons for such localisation may be

that these members register damage to these subcellular compartments, and effect changes to their functions, or effect cytoplasmic signalling eventually integrating these into the balance of apoptosis-related signals. On the other hand, the pro-apoptotic members localise predominantly to the cytoskeleton. The key function of anti-apoptotic Bcl-2 members appears to be resisting the release of pro-apoptotic signals from the mitochondria (Gross et al., 1999). The exact manner in which the opposing actions of the various Bcl-2 proteins balance out remains elusive. In *Caenorhabditis elegans*, the anti-apoptotic homologue CED-9 prevents the setting in of apoptosis through binding to and sequestering the Apaf-1 homologue CED-4 (Metzstein et al., 1998).

The key regulatory point where anti-apoptotic Bcl-2 protein acts appears to be the release of apoptogenic cytochrome-c from the mitochondria (Antonsson & Martinou, 2000). On the other hand it had been reported that cytochrome-c may be released through interaction with pro-apoptotic Bcl-2 with isolated mitochondria. It has not been fully elucidated as to how this can be effected but the following working hypotheses had been forwarded:

1. Bcl-2 members form channels in the mitochondrial membrane facilitating protein transport.
2. Bcl-2 members interact with other proteins to form channels. Some members can interact with voltage dependent anion channel.
3. Bcl-2 members induce rupture of the outer mitochondrial membrane.

It is assumed that the overall balance of pro- and anti-apoptotic Bcl-2 members would determine if the caspases may proceed with apoptotic missions. An important characteristic of Bcl-2 members, lending support to this belief, is that these proteins can

form heterodimers which may render opposing actions neutralised. The inhibition of the intrinsic pathway by Bcl-2 is usually bypassed by the death receptor pathway. The effects of the anti-apoptotic members of Bcl-2 proteins have been confirmed in cancers and cell cultures studies. Protection afforded by the Bcl-2 members applies to a range of cytotoxic drugs, gamma and ultra-violet irradiation; cytokine withdrawal, dexamethasone and staurosporine (Kulkarni and McCulloch, 1994).

Regulation at death receptor: FLIPs

The activation of procaspase-8 may also be regulated through inhibition. A class of proteins called FLIPs (FADD-like ICE inhibitory proteins) have been discovered to share similar sequence with procaspase-8 but lack the essential residues involved in catalysis (Shu et al., 1997; Irmeler et al., 1997). In some cases of death initiated through death receptors the exit of cytochrome-c into the cytosol is probably secondary to an overriding pro-apoptotic assembly of caspases and Bcl-2 members.

Regulation of apoptotic signals: IAP and caspase-3

Recently a family of genes encoding for proteins which confer inhibitory effects on caspases had been discovered. First isolated in baculovirus (Crook, Clem & Miller, 1993), homologues had been identified in humans (Roy et al., 1995; Rothe et al., 1995). These are the inhibitors of apoptosis IAP (Salvesen & Duckett, 2002). The IAP proteins act at the level of activated caspases eg. caspase-3 (Deveraux & Reed, 1999). IAPs exhibit high affinity binding to caspase-3 and mask the active site. Inhibition occurs through steric occlusion of the normal substrates of the caspase. This binding is reversible.

Regulation of apoptotic signalling prevents unwarranted cell death. Controls act at the level of signal integration via factors like the Bcl-2 proteins and FLIPs. These prevent the maturation of the initiator caspases, caspase-9 and caspase-8. Further downstream IAPs prevent the actions of the effector caspase-3. Apoptosis may therefore be modulated at its initiation as well as execution phases. It is hoped that with targeted manipulation at these points, death may be effected in cancer cells resistant to cytotoxic agents (Jansen et al., 1998; Waters et al., 2000); degenerative disorders such as cardiomyopathy may be delayed or reversed (Mocanu et al., 2000); severity of ischaemic injuries may be lessened (Endres et al., 1998); and sepsis and its systemic sequelae may be halted (Hotchkiss et al., 1999).

Phosphatidylserine: a membrane marker of apoptosis

In addition to the dramatic changes of pinching off through blebbing, the plasma membrane of an apoptotic cell also acquires distinct characteristics. Acquisition of some of these characteristics has been noted to be directly related to the activation of caspases (Martin et al., 1996; Naito et al., 1997). The best characterised change in the plasma membrane of an apoptotic cell is the externalisation of phosphatidylserine PS (Martin et al., 1995). Phosphatidylserine is an essential component of plasma membrane and probably serves to maintain membrane integrity. This anionic phospholipid is actively retained on the inner leaflet of the plasma membrane of normal cells through the actions of transporter-enzymes called translocases. The balance of inward and outward fluxes of PS is balanced by translocases and the equilibrium is tipped to this asymmetry in normal viable cells. During apoptosis additional activities of a protein called scramblase keeps the PS residues flipped to the outer leaflet of the plasma membrane (Frasch et al., 2000). Externalisation of PS is an event downstream

of activation of caspase-1, also known as interleukin-1-beta converting enzyme (ICE). Exposure of PS was shown to be prevented by the pan-caspase inhibitor Z-VAD linking it intimately to caspase activation (Naito et al., 1997). This caspase-dependent event was also noted to be independent of nuclear events in apoptosis. Preparations of enucleated cytoplasts displayed PS on the membranes when caspase-1 within these was activated (Martin et al., 1996). The externalisation of PS has also been shown to be an early uniform feature of apoptosis regardless of the initiating stimulus. Over expression of anti-apoptotic Bcl-2 members prevents the surfacing of PS externally (Martin et al., 1995). This indicates the exposure of PS is effected only after a cell is committed to apoptosis. This event usually precedes the loss of plasma membrane integrity by several hours as observed with in vitro studies.

Presently the exposure of PS on cell membranes is generally accepted as a feature of ongoing apoptosis (Martinez and Freyssinet, 2001). The exposed PS residues are marked by interaction with a molecule called Annexin-V. The binding of Annexin-V to cell membranes can be visualised through flow cytometry or immunofluorescent microscopy. Positive membrane binding for Annexin-V is one of the most commonly used criteria for identifying apoptotic cells.

Clearance of apoptotic cells

The restructuring of an apoptotic cell into membrane bound bodies sets the foundation for the next step in defining the apoptotic process. Apoptotic bodies necessitate disposal. It is generally accepted that this task may be undertaken by neighbouring cells or by tissue macrophages, in the capacity of non-professional and professional phagocytes respectively (Fadok, Bratton & Henson, 2001). In fact it has been proposed

that the crucial feature of apoptosis *in vivo* is that it leads to the recognition and engulfment of intact cells or membrane-bound apoptotic bodies by phagocytes (Savill et al., 2002). Observations in normal tissues typically find a paucity of apoptotic cells implying that apoptotic cells are normally expediently and efficiently cleared. The interface between an apoptotic cell and a macrophage is likely to implicate a host of reactions between signals and receptors (Hengartner, 2001; Ravichandran, 2003). There are probably several changes on the surface of a cell declaring apoptotic-self in contrast to normal-self (Savill, 2002; Krieser & White, 2002). Correspondingly there may be receptors on the macrophage specific for these alterations on apoptotic cells. The physiological milieu within which this interaction occurs may also supply factors in supplemental roles for this. It is generally accepted that following recognition and engulfment of apoptotic bodies, an immunosuppressive effect is elicited within macrophages (Savill, 1998; Savill & Fadok, 2000). Intense studies in the past decade have revealed much about the clearance of apoptotic cells and derangements in this process has been linked to many immunity and inflammatory disorders (Henson et al., 2001). It is anticipated that uncleared apoptotic bodies would eventually undergo necrotic degradation exposing a multitude of hitherto excluded cellular antigens. The auto-antibodies in the autoimmune disease systemic lupus erythematosus (SLE) reflect sensitisation of the adaptive immune system by auto-antigens like nucleic acids and other nucleoproteins (Casciola-Rosen et al., 1994; (Rosen and Casciola-Rosen, 1999). Interests in the factors that define the fate of apoptotic cells are immense, and it is hoped that some of the observations may soon translate to therapeutic principles.

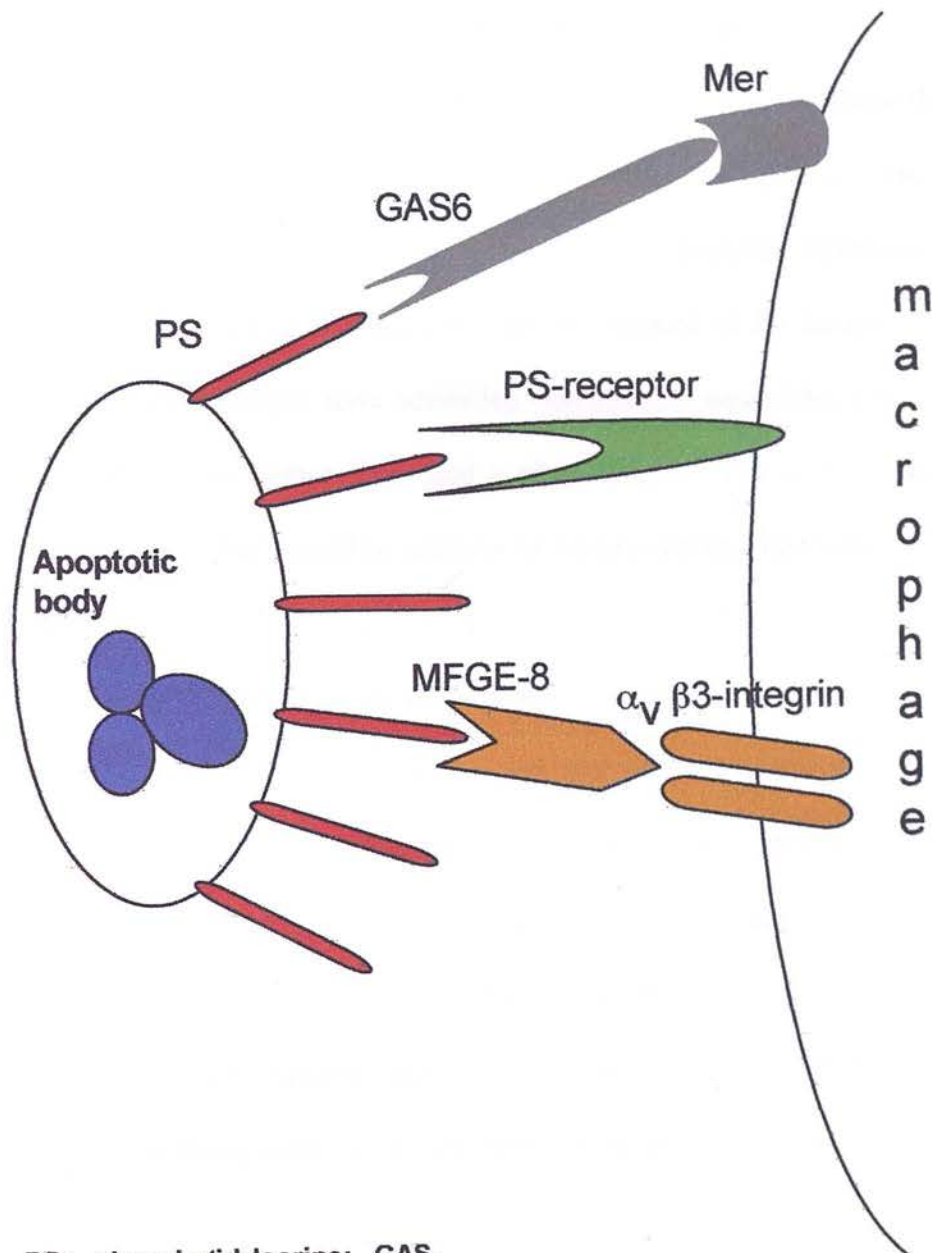
Among the signals on the apoptotic-self, some facilitate tethering to the phagocyte; some initiate engulfment, whilst others may mediate both attachment and engulfment

(Krieser & White, 2002). Several of these interactions have now been characterised. It appears that redundancy occurs with these interactions and no single signal possesses an overriding dominant effect. The central role of the macrophage as a professional tissue phagocyte has implicated the innate immune system as essential for this physiological outcome. This area of research is intense and new candidate ligand-receptor interactions are reported regularly. To date, several of the interface interactions have been well characterised. The examples elaborated below are not exhaustive but serve to illustrate the framework with which membranes on apoptotic cells are identifiable from normal membranes and how uptake of apoptotic cells is initiated.

Recognition of apoptotic cells: PS and PS receptor

Phosphatidylserine (PS) residues externalised on the plasma membrane is one of the most extensively studied characteristics which is central to the interaction between apoptotic cells and the macrophage (Fadok et al., 2001; Fadok et al., 1992) (Figure 1.2). PS is recognised by both macrophages and non-professional phagocytes. A putative receptor on the macrophage has in fact been cloned (Fadok et al., 2000). This PS receptor was recently demonstrated to be pivotal in mediating engulfment (Hoffman et al., 2001). Ligation of PS receptors in macrophages to PS vesicles and PS-coated cells was crucial in stimulating uptake through macropinocytosis. However, PS did not appear to be significantly involved in tethering apoptotic particles to macrophages, as PS residues alone promoted poor binding of the particles to macrophages. Mere tethering was differentiated from engulfment by feeding red blood cells together with various antibodies and ligands to macrophages followed by lysing those which had not been ingested. Most of the other known putative receptors, when exposed to their respective ligands on the red blood cells did not result in engulfment.

Figure 1.2 Phagocytosis of apoptotic cell involving phosphatidylserine residues. The receptors on phagocytes are indicated together with bridging moieties.



PS: phosphatidylserine; **GAS-6:** Growth-Arrest-Gene protein 6; **MFGE:** Milk Fat Globule-EGF-factor 8 protein;

The role of the PS receptor in phagocytosis is further supported by *in vitro* observations that reducing the expression of the PS receptor in phagocytes, by transfection with small-interfering RNA, decreased phagocytosis of apoptotic targets (Wang et al., 2003). *In vivo*, however, the evidence is less conclusive (reviewed by Williamson and Schlegel, 2004). Murine knockouts of the PS receptor gene (*psr*) display various developmental defects in the lungs and brain (Li et al., 2003), as well as in erythropoietic and T-lymphocytic lineages (Kunisaki et al., 2004). These defects were attributed to reduced clearance of apoptotic cells by macrophages in *in vivo* assays. In contrast, Bose et al. (2004) observed no defect in apoptotic clearance in the *psr* knockout mouse. It has been suggested that the method of PS receptor isolation by Fadok et al. (2000) might have identified non-specific candidates (Williamson and Schlegel, 2004). The authors had used a phage display library probed with the PS receptor antibody, which could be sensitive to weakly-reacting epitopes.

PS is also exposed on the cell membranes of necrotic cells, through membrane permeabilisation, and would be recognised and processed by phagocytes. It was observed that macrophages bound and engulfed necrotic and apoptotic cells to similar extents and with similar saturable kinetics (Cocco & Ucker, 2001). Binding and engulfment of both apoptotic and necrotic cells by macrophages appeared to be dependent on PS. This suggests that recognition of PS exposure by macrophages is a not a unique initiating event for phagocytosis of apoptotic cells, as necrotic cells also utilise this mechanism.

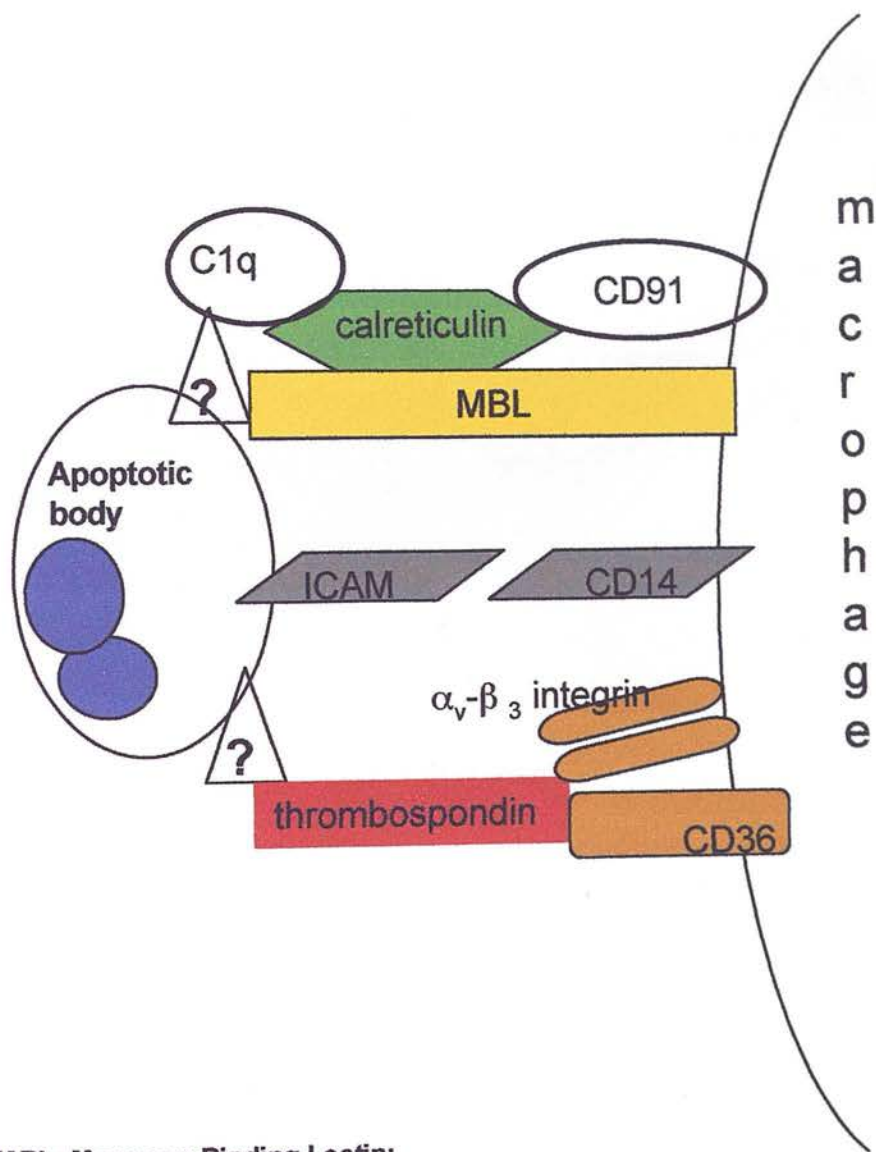
In conclusion, the functions of the PS receptor in phagocytosis of apoptotic cells remain elusive. A further complicating observation localises the PS receptor in *Hydra*

to the nucleus instead of the cell surface (Cikala et al., 2004). Nuclear localisation signals in the receptor protein sequence appear to be conserved in several species, including those in mammalian, *C. elegans* and *Drosophila* homologues. This finding poses a challenge to the role of the PS receptor as a cell surface mediator of phagocytosis.

Other receptors recognising PS

Phosphatidylserine may also be recognised by other receptors (Figure 1.3). A member of the receptor tyrosine kinase family Mer has been reported to be involved in phagocytosis of apoptotic cells. It is expressed in phagocytes and this represents a novel function for the molecule. The phagocytic deficiency, on knockout of the Mer gene, was restricted to apoptotic cells whilst Fc receptor-mediated phagocytosis or particle ingestion was unaffected (Scott et al., 2001). It was found that whilst the macrophages from mer-knockout mice showed deficiency in the engulfment of apoptotic thymocytes. The macrophages were, however, equally competent in ingesting *Listeria monocytogenes* as compared to wild-type macrophages, pointing to a role for mer in the recognition of apoptotic cells. The mer tyrosine kinase probably uses GAS6 (growth arrest specific protein 6) as a bridge to phosphatidylserine (Nakano et al., 1997). Mer has been shown to bind to GAS6 whilst GAS6 has been demonstrated to bind to phosphatidylserine residues on apoptotic cells. The ligand for mer might be phosphatidylserine on apoptotic cells via a bridging interaction between PS and GAS6 to mer. It was suggested that the cytoplasmic domain of the mer receptor may trigger the engulfment mechanism in macrophages.

Figure 1.3 Interaction using non-phosphatidylserine residues. Receptors on the macrophages involved in phagocytosis are indicated; with unidentified ligands on the apoptotic cell.



MBL: Mannose Binding Lectin;
ICAM: Inter-Cellular Adhesion
Molecule;

 indicates uncharaterised entity

The secreted milk fat globule-EGF-factor 8 protein (MFGE-8) was reported to link apoptotic cells to phagocytes (Hanayama et al., 2002). It is produced and secreted by thioglycollate activated macrophages and binds to aminophospholipids such as phosphatidylserine. This binding competed with that of Annexin V. When added to fibroblasts expressing high level of $\alpha_v\beta_3$ integrin, phagocytosis of apoptotic cells was greatly promoted. Therefore it appears that MFGE-8 recognises PS residues on apoptotic cells and bridges these to phagocytes expressing the vitronectin receptor ($\alpha_v\beta_3$ integrin). The display of PS on the surface, a uniform feature on apoptotic cells, therefore seems to be recognised by several receptors thus enhancing the expedient identification of these by phagocytes. Whilst PSR appears to be specific in mediating engulfment many other receptors would appear to facilitate the process by maximising the juxtaposition and tethering of apoptotic bodies to phagocytes.

CD36, a member of the class B scavenger receptors on macrophages, was one of the first to be implicated in recognition of apoptotic cells (Savill et al., 1990; Savill et al., 1992). CD36 was shown to interact with the vitronectin receptor ($\alpha_v\beta_3$ integrin), expressed on the surface of leukocytes, and this cooperation employed thrombospondin as a molecular bridge to recognise apoptotic cells. The factor on the apoptotic cell being recognised has not been identified (Moodley et al., 2003). CD36 is also involved in endocytic clearance of oxidized low-density lipoprotein (oxLDL) present on the surfaces of apoptotic cells (Chang et al., 1999; Kagan et al., 2002). Apparently, oxidation of the membrane phospholipids results in oxLDL-like sites on apoptotic cells, which is required for recognition by macrophages.

Class A scavenger receptors on macrophages and other phagocytes have also been implicated in the recognition of apoptotic cells (Platt et al., 1996). The asialoglycoprotein receptor on hepatocytes can function in the ingestion of apoptotic cells (Dini et al., 1992). This is consistent with the observation that apoptotic cells lose sialic acid residues from complex carbohydrates on their surfaces (Duvall et al., 1985).

Interestingly, apoptotic cells can switch repulsive signals which are recognised by receptors on phagocytes into non-repulsive signals. One such molecule involved in this switch is CD31, which has been reported to mediate binding and ingestion of apoptotic cells by macrophages (Brown et al., 2002). In viable leukocytes, CD31 mediates detachment from phagocytes in viable leukocytes. However, this CD31-dependent activity is absent in apoptotic leukocytes, promoting tethering of apoptotic cells to phagocytes and possibly aiding ingestion.

Recognition of apoptotic cell: innate receptors

A host of pattern recognition receptors are employed in the innate immune system to recognise non-self antigens such as those of microbes. There is evidence to suggest a role for these in the recognition of apoptotic cells. One such candidate is the lipopolysaccharide (LPS) receptor CD14 on macrophages utilised to bind gram negative bacterial antigens. It has been implicated in the recognition and engulfment of apoptotic cells (Devitt et al., 1998). Using a monoclonal antibody which binds to the surface of macrophages and specifically inhibits interaction with apoptotic cells, the authors confirmed the epitope recognised to be part of CD14. The region of CD14 implicated was close to that which recognised LPS. The anti-inflammatory response to apoptotic cells however was preserved upon ligation of CD14. It was therefore

suggested that distinct downstream responses were possible with CD14. The ligand for CD14 on apoptotic cells however has so far not been identified, though ICAM-3 is a possible candidate (Moffatt et al., 1999).

The innate immune system with the employment of pattern recognition receptors seems well poised to play a major part in the clearance of apoptotic cells. The nonclonal system of recognition would fit well with the demand of a disposal system for cells undergoing physiological death (Medzhitov & Janeway 1997). This system is mainly executed by the evolutionary conserved family of Toll-like receptors on macrophages (Medzhitov, 2001), which recognize bacterial antigens and activate downstream pro-inflammatory events (Underhill & Ozinsky, 2002). However there has not been any conclusive evidence of the involvement of Toll-like receptors in the recognition and uptake of apoptotic cells. Toll-like receptors appear unlikely to be utilized in the recognition and phagocytosis of apoptotic cells (Blander & Medzhitov, 2004). However, the matter may be more complicated. The close associate of Toll-like receptor, CD14, which binds bacterial lipopolysaccharide (LPS) has been implicated in the recognition of apoptotic cells, albeit at a site not identical to the LPS-binding moiety (Devitt et al., 1998).

Innate recognition: a role for serum factors

The complement system of the innate immune system had also been shown to have a role in the clearance of apoptotic cells. A member of the first component in the classical pathway, C1q has been linked to uptake of apoptotic cells. Deficiency in this protein is associated with autoimmune diseases. C1q knock-out mice demonstrate features of systemic lupus erythematosus (SLE). The complement factor C1q binds to the blebs

on apoptotic cells. Incubating C1q with apoptotic cells maintained in serum-free conditions facilitated uptake by phagocytes (Ogden et al., 2001).. It was found that C1q engaged surface calreticulin (CRT) on phagocytes. This surface CRT, a calcium sequestering chaperone is associated with CD91, an endocytic receptor protein on the phagocyte membrane. Upon interaction with C1q the complex initiated uptake of apoptotic cells through macropinocytosis. In the absence of C1q this targeting toward apoptotic cells would be less efficient. The apoptotic bodies would likely undergo necrotic lysis releasing self antigens which were normally excluded from the immune response. This therefore might be a key event in the initiation of autoimmune response. In the same study, Ogden et al. also reported similar observations with mannose-binding lectin (MBL). MBL is a member of the collectin family which binds specific pathogen-associated molecules such as those on bacteria. They concluded that uptake of apoptotic cells involved pattern recognition molecules of the innate immune system. The moiety on the blebbing membrane of apoptotic cells recognised by C1q or MBL had yet to be characterised.

The antigen BOB78: a surface marker of apoptotic cells

A novel monoclonal antibody called BOB78 isolated in the Lister Research Laboratory was found to identify apoptotic neutrophils through membrane binding. Following inoculation of mice with human leukaemic myeloid THP-1 cells, hybridomas using the splenic cells were cultured. From the panel of retrieved antibodies BOB78 was found through flow cytometry and indirect immunofluorescence to identify apoptotic cultured neutrophils. BOB78 is an IgM isotype antibody. The population of neutrophils identified was thought to be in late apoptosis (Hart et al., 2000). It was found that the BOB78 antigen was normally present in the cytoplasm of neutrophils. The quantity of

BOB78 antigen within neutrophils did not change dramatically upon apoptosis. It appears that BOB78 is a cytoplasmic antigen which translocates to the plasma membrane in apoptotic cells.

There had been repeated attempts to purify the BOB78 antigen through immunoprecipitation; however, it had proved difficult. The proteolytic activities within apoptotic neutrophils might have contributed to this difficulty in spite of the fact that the BOB78 antigen is present in the cytoplasm normally. The titre of BOB78 antibody in the supernatants of cultured hybridomas might also have been a limiting factor.

Aims of the project

The studies undertaken were aimed at elucidating the nature of the antigen identified by the monoclonal antibody BOB78.

With the knowledge that BOB78 identified a population of apoptotic neutrophils as reported earlier (Hart, Dransfield et al., 2000), further characteristics of BOB78 which attracted particular attention were:

1. Is the BOB78 antigen a common feature on most apoptotic cells?
2. At which stage of apoptotic changes does the antigen recognised by BOB78 appear on the membrane of cells?
3. What is its normal turnover and location in cells
4. The nature of the BOB78 antigen as determined through sequencing

The studies I had carried out had been targeted to addressing these questions with the ultimate aim of determining the characteristics of the molecule identified by the BOB78 antibody.

CHAPTER 2

METHODS

(see Appendix 1 for materials)

Cell Culture & Maintenance

In vitro cultures of various cell lines were maintained in standard conditions of commercial medium supplemented with 5 to 10 per cent by volume of foetal calf serum (FCS). The cultures were incubated at 37°C in humidified air with 5% carbon dioxide. Leukaemic cell lines which included the monocytic THP-1 and megakaryocytic MEG-01 were maintained in RPMI (GibcoBrl); whilst epithelial cell lines hepatoma HUH-7 and pancreatic Mia were maintained in DMEM (Dulbecco's Modified Eagle's Medium, GibcoBrl). THP-1 and MEG-1 cells were usually maintained in log-phase at a density of $8 - 10 \times 10^5$ cells/ml in medium, and subcultured by diluting 1/3 in fresh medium every 2 days or as indicated by the change in pH of the medium. HUH-7 and Mia cells which were grown in monolayers were subcultured after detachment through trypsinisation when the cells reached confluency. 0.05% trypsin in 2mm EDTA was added to the cell monolayers after 2 washes in PBS (phosphate buffered saline). After incubating with 10mls of trypsin per 75cm² flask for 30 seconds at 37°C, the trypsin was decanted leaving only a thin film. A further incubation of 5 minutes usually sufficed to detach the cells when these were easily dislodged with sharp taps to the side of the flasks. The activity of trypsin was aborted with fetal calf serum, and the detached cells were suspended in 10% FCS / DMEM. After two further washes in 10% FCS/DMEM the cells were plated 1/3 – 1/5 onto the flasks, which would usually keep the cells in log phase of growth.

A. *Induction of apoptosis*

Human leukaemic THP-1 cells were diluted to 1×10^6 cells/ml in fresh medium, and synchronised through S-phase arrest by incubating with 2mM thymidine for 16 hours. The cells were released from the thymidine block by washing in large quantities of RPMI at least twice. The cells were then resuspended at 1×10^6 cells/ml in RPMI/10%FCS. Apoptosis was induced with 3 μ M camptothecin (CPT, Sigma) in culture, with typically 15 to 20 per cent of cells apoptotic by 6 hours.

B. *Measuring cellular DNA content with propidium iodide on flow cytometry*

Cells were collected from culture and washed in cold PBS through centrifugation at 1000g for 5 minutes at room temperature. To each tube was pelleted 1×10^6 cells which were resuspended in 1ml of nuclear staining solution containing propidium iodide in sodium citrate and Nonidet 40 (refer to appendix for materials). To each tube was then added 1 ml of RNase solution. The reaction was incubated in the dark for 30 minutes at room temperature. The tubes were then processed through cytometry using FL3 to read off fluorescence from propidium iodide bound to cellular DNA.

C. *Neuraminidase digestion of cells*

Human leukaemic THP-1 cells were harvested, washed and resuspended to a concentration of 10×10^6 cells/ml in normal saline (154mM NaCl). Neuraminidase-X, a deglycosylating agent isolated from *Clostridium perfringens* (Sigma), was added to the THP-1 cells to a concentration of 0.1U/ml. Cells were incubated for at least 30 minutes at 37°C with constant mixing. The cells were washed with PBS/BSA1% as before prior to immunostaining for flow cytometry.

D. *Tunicamycin inhibition of N-linked glycosylation*

Human THP-1 cells were harvested and maintained in fresh culture medium of RPMI/10% FCS at a density of 1×10^6 cells/ml. Cells were incubated with neuraminidase-X (*Clostridium perfringens*, Sigma) at a concentration of 0.1U/ml for at least 1 hour at 37°C in 5% CO₂ atmosphere. Cells were then washed in medium before returning to culture in tunicamycin concentrations ranging 0 – 100µg/ml (Sigma) for 18 hours. The cells were then harvested and washed and processed for immunolabelling for flow cytometry.

E. *Inhibition of biosynthesis-secretory pathway*

Human leukaemic THP-1, hepatome HUH-7 and pancreatic Mia cells were cultured and treated with monensin or Brefeldin-A. Monensin is a Na⁺ ionophore which blocks glycoprotein secretion. Brefeldin-A is a fungal metabolite which disrupts the structure and function of the Golgi apparatus (Sigma catalogue).

Immunocytochemistry

Cells were either grown in chamber slides or centrifuged onto adhesive microscopic slides. Cells were washed in PBS and drained well. Fixation was achieved by immersion in a large volume of cold methanol (previously kept at –20 C) for 10 -15 minutes on ice. Slides were either rinsed in PBS or washed in a PBS bath for 20 minutes. Primary antibodies were added at 100µl per chamber or 50 – 100µl per slide, and incubated for about 60 minutes at room temperature. Slides were then washed in PBS for 10 minutes. A total of 3 washes were performed before the addition of secondary antibodies. Aliquots of 100µl per chamber, or 50 – 100µl per slide, of secondary antibodies eg. FITC and TRITC-conjugated antibodies (DAKO) were added,

used at 1:20 and 1:50 dilution respectively. Slides were then incubated in the dark for 60 minutes at room temperature, then washed 3 times in PBS as before. Nuclear counter-staining was performed with 100µl per slide of TO-PRO 3 (4µl/ml dilution, Cambridge Bioscience) for 5 – 10 minutes at room temperature. Finally, slides were rinsed in distilled water, mounted with a cover slip using fluorescent mounting medium (DAKO) and kept in the dark until the mounting medium dried.

Flow Cytometry

A. *Detection of surface antigens*

Cells grown in suspension eg. THP-1 were used. The cells were collected from suspension culture by centrifugation (room temp or 4°C) at 550g for 5 minutes. Half to one million cells in a single tube were stained with 1µg of primary antibodies. The cells were washed twice with phosphate buffered saline containing 1% bovine serum albumin (PBS / 1%BSA) and collected into a pellet at 550g for 5 minutes. Blocking of non-specific background staining was effected with 20% normal rabbit serum in PBS for at least 20 minutes at room temperature (or 4°C). Primary antibodies at 1 µg per 50µl of PBS / 1%BSA were added to $0.5 - 1 \times 10^6$ cells and incubated for at least 30 minutes at room temperature (or 60 minutes at 4°C). The cells were washed twice with 1ml of PBS / 1%BSA per tube to remove the unreacted primary antibodies. Secondary antibodies (eg. PE and FITC-conjugated antibodies) at 0.5 µg / 50µl were added per tube and incubated for at least 30 minutes at room temperature (or 4°C). After this the cells were washed twice as before and resuspended in 1% formaldehyde in PBS at 1ml per tube (ie. $0.5 - 1 \times 10^6$ cells).

Cells were analysed by the Coulter EPICS XL Cytometer using the ExpoTM32 ADC Analysis program. FITC was excited by a 15mW argon laser at 488nm, and detected in the FL1 channel using a 525 bandpass filter with a detection range of 505-545nm. PE was detected in the FL2 channel using a 575 bandpass filter with a detection range of 560-590nm.

B. Detection of intracellular antigens

To permeabilise the cells for intracellular staining, cells were pelleted at 550g for 5 minutes. The medium was decanted off carefully and completely, and cold 4% paraformaldehyde was added. Cells were kept in melting ice for at least 15 minutes and pelleted. Paraformaldehyde was decanted off and cold methanol (100% kept at -20°C) was immediately added. Cells were kept in melting ice for 30 minutes, pelleted, and washed twice with PBS. The cells were then blocked in 20% rabbit serum as described above (Pollice et al., 1992; Tsan et al., 2000).

C. Surface and nuclear staining of cells undergoing apoptosis

For surface staining of cells undergoing apoptosis, staining and washings were performed at 4°C. Fixation with 1% paraformaldehyde / PBS was omitted. Cells in each tube were resuspended in 100 µl of 0.02% propidium iodide (2 µg/ml) in PBS no more than 5 minutes before flow cytometry. Propidium iodide staining was detected in the FL3 channel using a 620 bandpass filter.

D. Fixation and immunolabelling of erythrocytes

Fixation

Erythrocytes were washed in PBS and resuspended in 100µl PBS. 1ml PBS containing 50µg/ml sodium dodecyl sulphate (SDS) and 1mg/ml bovine serum albumin (BSA) was added. After 1 minute, 9.7ml PBS, 0.3ml formalin (37% formaldehyde in PBS) and 10µg/ml SDS were added. After 90 minutes, 0.8ml formalin was added and left at room temperature overnight. The cells were washed in PBS and the procedure was repeated without the addition of BSA. The fixed cells were finally washed in PBS and resuspended in a concentration of 2×10^7 cells/ml PBS containing 5mg/ml BSA and 0.1% sodium azide.

Immunolabelling

50µl (10^6 cells) of fixed erythrocytes was added to the FITC or R-PE-labelled antibody solution. Cells were incubated for 1 hour at room temperature in the dark and washed once in PBS containing 1% BSA before immunodetection by flow cytometry.

Immunoprecipitation with BOB78 antibody

A. Preparation of cell lysate

Cells were washed once with cold PBS and pelleted by centrifugation. Cells were lysed by adding TENT lysis buffer (10mM Tris-HCL, 5mM EDTA, 150mM NaCl, 1% Triton-X 100, and freshly added protease inhibitors cocktail), mixed well through pipetting and left to incubate for 30 minutes on melting ice in the cold room. The mixture was then centrifuged at 12,000g for 10 minutes to clarify the lysate, pelleting

out undigested membranes and nuclei. The supernatant was carefully harvested and passed through a 23G needle for 3 times to shear any DNA present in the lysate. Measurement of protein concentration was performed with the Biorad kit using albumin dilutions as standards, and absorbance at 590nm was measured using a Bio-Rad spectrophotometer.

B. Immunoprecipitation: incubation of lysate with antibodies

The cell lysate for immunoprecipitation was precleared of non-specific binding by incubating with protein A Sepharose beads for 1 hour on ice. Prior to this, the beads had been collected by centrifuging at 150g for 30 seconds and washed once with excess lysis buffer containing protease inhibitors. 10µl of beads was added to 100µl of cell lysate (about 500µg protein). Meanwhile fresh beads were washed as above and most of the washing lysis buffer was removed. These were then conjugated to a bridging antibody by adding 10µl (=10µg) of rabbit anti mouse IgM (DAKO), and incubated on melting ice for 2 hours in the cold room. Next 20µl of the conjugated beads was incubated with 200µl of anti-BOB78 supernatant (IgM variety) on melting ice for 2 hours to capture the IgMs. The IgM-loaded beads were then incubated with the precleared lysate overnight in the cold room on ice.

C. Collecting the immunoprecipitates

The beads were collected by centrifuging at 350g for 30 seconds. These were washed 3 times, each time by pelleting at 350g for 30 seconds, resuspending in 500µl of lysis buffer containing protease inhibitors, and agitating for 10 minutes on a rotating platform. The immune complexes were released from the beads by incubation with 20µl of 5X loading buffer (2% SDS) and boiling at 95° C for 5 minutes. The proteins

were then separated on a 12% SDS polyacrylamide gel which was run at 200V for about 75 minutes.

One half of the gel was stained with colloidal Coomassie Blue (Gelcode) while the duplicate half was blotted onto nitrocellulose membrane. Immunoreactions on the Western blots were performed as described below. The transferred antigens were probed with antibodies including BOB78 antibody.

Western blotting

A. Denaturing protein electrophoresis and blotting

Protein samples were prepared to contain 1/4 volume of the 4X loading buffer which included 10% sodium dodecyl sulphate and 5% 2-beta-mercaptoethanol (see appendix under Protein electrophoresis and Western blotting). The samples were denatured by heating at 95°C for 5 minutes. 15µg of protein and appropriate molecular weight markers were loaded in each lane of a 12% SDS-polyacrylamide gel. Electrophoresis was performed at 200V for approximately 75 minutes to attain good separation of the molecular weight markers.

Nitrocellulose membrane and sandwich blotters (cut to size of the gel) were prepared for blotting by soaking in transfer buffer. The stacking portion of the SDS-polyacrylamide gel was cut away and discarded. The nitrocellulose membrane was applied onto the separating portion of the gel, sandwiched between 3 pieces of blotters on each side. Transfer was achieved with the membrane orientated towards the anode

end of a semi-dry transfer apparatus moistened with distilled water, run at a constant current of 80 mA. This typically took 55 minutes for effective and complete transfer. The transferred proteins were stained with Ponceau, and lanes as well as molecular weights were marked with carbon pencil.

B. Immunodetection of proteins on Western blot

After blotting, the nitrocellulose membrane was incubated in blocking solution made of 5% milk proteins (w/v) in TBS (Tris-buffered saline) for at least 20 minutes at room temperature. The membrane was transferred into solutions of primary antibodies diluted in TBS/0.025% Tween 20 with 5% milk proteins (eg. 1:100 dilution of BOB78), and incubated overnight at 4°C with gentle rocking. It was then washed 3 times in TBS/0.025% Tween 20, and incubated with peroxidase-conjugated secondary antibodies diluted in TBS/0.025% Tween 20 with 5% milk proteins (eg. rabbit anti-mouse at 1: 1500 dilution) for at least 1 hour at room temperature. This was followed by 3 washes with TBS/0.025% Tween 20 and 2 further washes with TBS. The immunoreaction was detected by enhanced chemiluminescence (ECL Western blotting analysis system, Amersham Pharmacia Biotech) using X-ray film.

Mass Spectrometry Analysis

Individual gel slices which were not present in control immunoprecipitations were cut from colloidal Coomassie Blue-stained gels, the protein eluted and then digested with trypsin (Promega). Peptides from the digested bands were then run on LC-MS and MALDI-MS mass spectrometers. The LC-MS consisted of a Famos autosampler, Switchos column switching unit and an Ultimate nanoLC (Dionex).

For the MALDI-TOF analysis aliquots of 0.5ml digests were mixed with 0.5ml α-cyano-4-hydroxy cinnamic acid (CHCA) matrix on a MALDI sample plate. The samples were then analysed on a Voyager DE-STR MALDI-TOF MS (Applied Biosystems) and the processed spectra searched against the NCBI non-redundant database using Protein Prospector. This analysis was carried out by Dr. Andy Cronshaw at the Edinburgh Protein Interaction Centre (EPIC) facility.

Enzyme-Linked Immunosorbant Assay (ELISA)

The titre of BOB78 antibody in culture supernatants was estimated with ELISA (enzyme-linked immunosorbant assay). This is essentially a chromogenic detection of antibody-antigen interaction, and can be used to measure the quantity of antigen present. ELISA was carried out using 96 well-plates. To each well was added 100µl of rabbit anti-IgM antibodies diluted to about 10mg/L with coating buffer. The plate was left to coat with the antibodies overnight. The plate was then washed with washing buffer three times. 100µl of the BOB78 supernatants and standards (5 dilutions) were added to each well to incubate for 2 hours at room temperature. The wells were then washed three times as before. To each well was then added 100µl of peroxidase-conjugated anti-mouse antibodies and incubated for 1 hour. The wells were washed and the chromogenic substrate 1,2-phenylenediamine dihydrochloride (tablets dissolved in 0.08% hydrogen peroxide in distilled water) was added. The development of colour was halted by addition of 150µl 1M H₂SO₄ to each well. The colour developed in the wells was then read with a photometer plate reader at 492nm. The quality of the estimation and the specificity of the colour development were checked from the curve generated with the standards, as well with blanks or standard dilutions loaded between supernatant-containing wells.

Replicates

All experiments were repeated on at least three separate occasions before incorporation into the study.

CHAPTER 3

CHARACTERISATION OF BOB78 ANTIGEN IN CELLS UNDERGOING APOPTOSIS

INTRODUCTION

Apoptosis and its progression in cells can be studied using a variety of techniques. Typical morphological changes associated with apoptosis are generally characterised by microscopy (Wyllie, Kerr & Currie, 1980; Willingham, 1999). DNA fragmentation is usually demonstrated by gel electrophoresis. Degradation of genomic DNA into internucleosomal segments results in residues of multiples of 200 base pairs rendering a typical ladder pattern on electrophoresis. Immunological-based techniques are widely used to identify membrane changes, as well as signalling pathways and other cytosolic events such as proteolysis of caspases. In addition, the analysis of several key parameters in apoptotic cells is possible through flow cytometry which has several advantages (Darzynkiewicz et al., 1992; Vermes et al., 2000). In particular, the dynamic state of apoptosis and cellular changes can be followed relatively easily. Cytoplasmic events such as the loss of mitochondrial transmembrane potential (MTP), release of cytochrome-c, and activation of caspases can be traced with specific ionic fluorochromes or fluorescent antibodies. When apoptotic cells are treated with nucleic acid-binding fluorescent dyes such as propidium iodide, a typical peak or plateau is detectable in the sub-G1 region. This corresponds to a hypo-diploid state as small DNA fragments leak out of cells during *in vitro* processing. Cell membrane changes such as the exposure of phosphatidylserine (PS) residues on the outer plasma membrane can be

identified with fluorochrome-conjugated Annexin-V (Vermes et al., 1995; van Engeland et al., 1998). Morphological changes in cells undergoing apoptosis, such as reduced size and increased granularity, are reflected by reduced forward angle scatter (FS) and increased side angle scatter (SS) respectively on flow cytometry. Apoptotic bodies which form as apoptosis progresses exhibit both reduced FS and SS. Cells which have ruptured would give rise to very small debris, detected as particles with extremely reduced FS. A proportion of cells in late apoptosis or cells which have undergone secondary necrosis would also be permeable to the fluorescent nuclear dye propidium iodide, enabling these to be separated from viable and apoptotic cells which maintain membrane integrity. This gives rise to populations of cells which are propidium negative (viable), propidium dim (apoptotic) and propidium bright (permeable/necrotic) (Nicoletti et al., 1991; Del Bino, 1999).

This chapter describes the characterisation of BOB78 antigen as an early marker of apoptosis and its localisation to the membrane blebs of apoptotic cells. Studies were performed using flow cytometry and fluorescence microscopy.

RESULTS

3.1 Characterisation of BOB78 antigen in viable cells

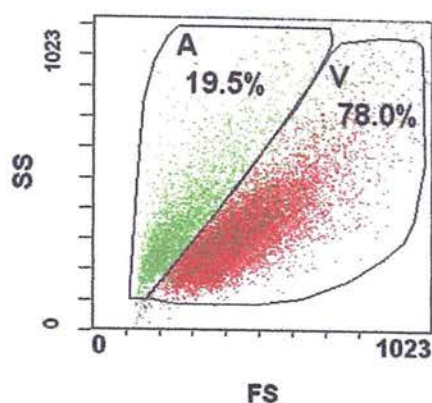
It has been reported that the BOB78 antigen is expressed on the surfaces of neutrophils in late apoptosis, where its detection is delayed temporally compared with other classical markers of neutrophil apoptosis eg. CD16. The BOB78 antigen has also been detected in intracellular compartments of normal neutrophils, possibly localised in granular compartments (Hart et al, 2000). This pattern of expression of BOB78 antigen

in cells was investigated with cultured human leukaemic THP-1 cells. THP-1 cells cultured in standard conditions were harvested and processed for flow cytometry as described in Methods (chapter 2). Additionally the THP-1 cells were also fixed and permeabilised to allow detection of intracellular antigens.

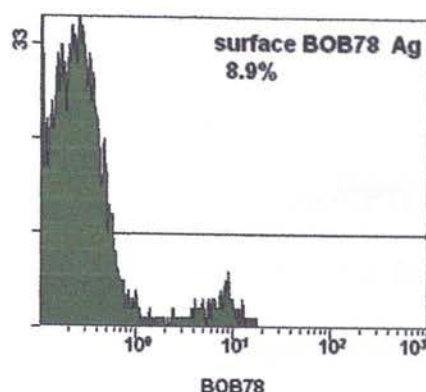
3.1.1 BOB78 antigen is expressed in the cytoplasm but not on the surface of viable THP-1 cells

Flow cytometry analysis showed that viable THP-1 cells cultured in standard conditions did not express BOB78 antigen on the surface. The small percentage of naturally dying THP-1 cells in the cultures however expressed BOB78 antigen on the surface. These cells were distinguished by the smaller cell sizes, with either unchanged or increased granularity as reflected by the side angle scatter on flow cytometry (figure 3.1). On fixation and permeabilisation to access intracellular antigens, BOB78 antigen was found to be expressed within normal THP-1 cells. It was interesting to note the positive fluorescence for BOB78 exhibited on the surface of dying THP-1 cells was to the same degree as that for intracellular BOB78 antigen. This however cannot be taken to infer that the pool of intracellular BOB78 antigen was mobilised onto the surface of dying THP-1 cells, as the process of fixation and permeabilisation may alter the quantity of the BOB78 antigen or the detection of it thereof.

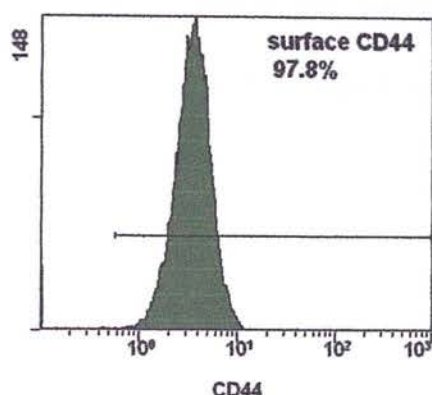
Figure 3.1 Expression of BOB78 antigen within untreated cells. Human leukaemic THP-1 cells were processed for flow cytometry, for surface as well as intracellular expression of BOB78 antigen. The secondary anti-mouse antibodies were conjugated with FITC.



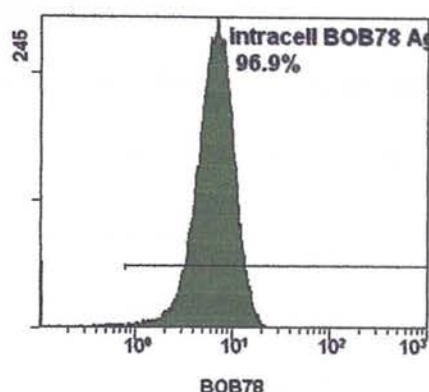
Dying THP-1 cells
are smaller



Half of the dying cells
express surface BOB78
antigen



Viable THP-1 cells
expressing CD44 on
surface



Permeabilised THP-1 cells
with intracellular BOB78
antigen

The BOB78 antigen is expressed within untreated cells. The BOB78 antigen is expressed on the surface of dying cells. Viable cells however do not normally express BOB78 antigen on the surface. The presence of BOB78 antigen within untreated cells is confirmed upon permeabilising untreated cells. The quantity of BOB78 antigen, reflected by the relative fluorescence, appears unchanged between viable cells and dying cells.

3.2 Characterisation of BOB78 antigen in apoptotic THP-1 leukaemic cells by flow cytometry

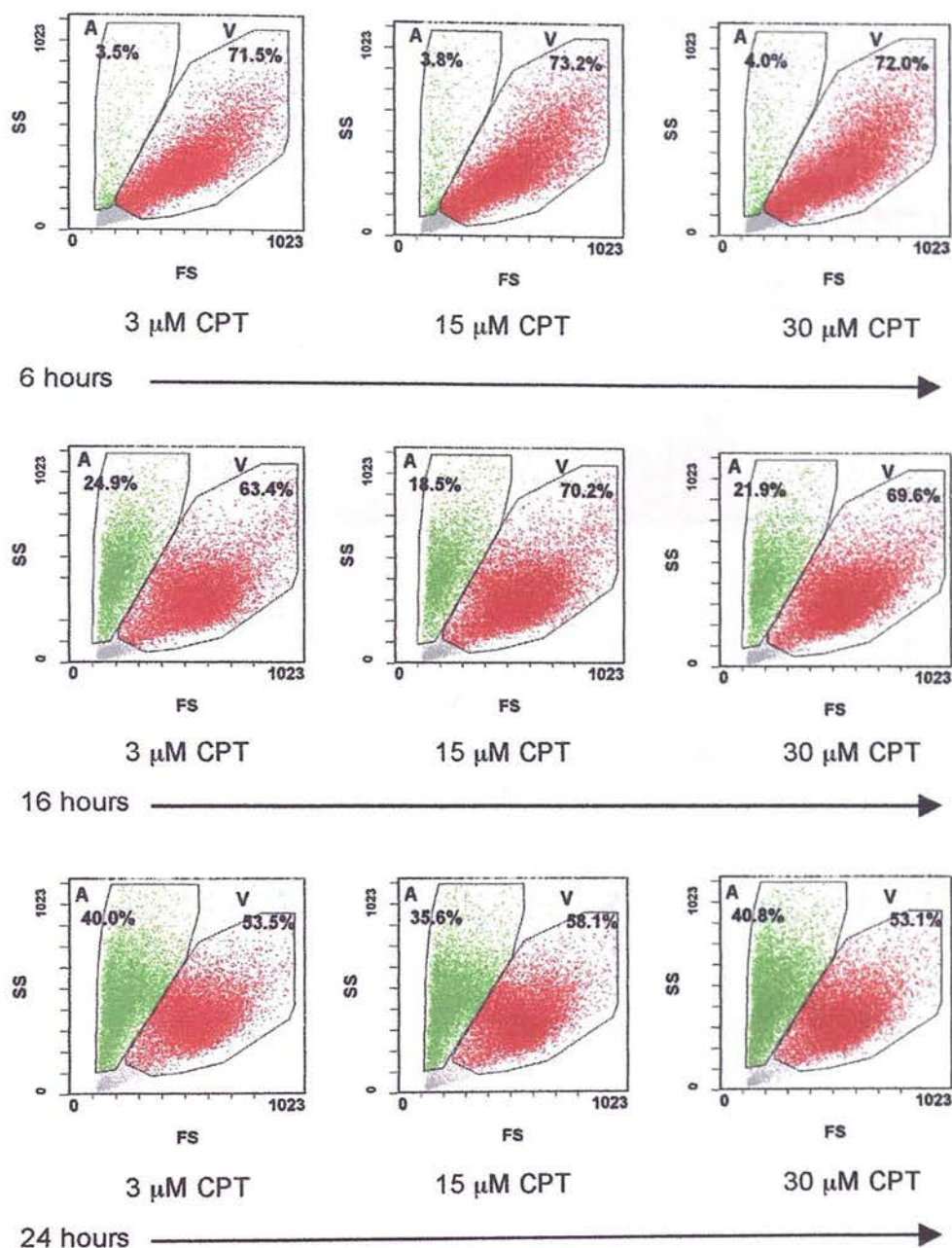
Method 1. Induction of apoptosis through treatment with camptothecin

The binding of the monoclonal antibody BOB78 to apoptotic cells was investigated using the human leukaemic cell line THP-1. Apoptosis was induced in THP-1 cells with the DNA topo-isomerase inhibitor camptothecin. Camptothecin (CPT) acts in the S-phase of the cell cycle and induces apoptosis in susceptible cells (Del Bino, 1991). Titration of camptothecin over a dose range from 3 to 30 μ M showed that the proportions of cells undergoing apoptosis were relatively constant (figure 3.2).

The proportion of THP-1 cells entering S-phase was increased using thymidine block. Thymidine acts through competitive quenching of other nucleic acids during DNA synthesis and leads to cessation of cell division. Arrest in S-phase of the cell cycle through 18-hour incubation with 2mM thymidine increased the proportion of the cells in S-phase. After the 18 hour incubation the proportion of dying cells remained comparable to the untreated population (figure 3.3). Release of the cells from the thymidine block was done by washing in medium and returning to standard culture conditions of RPMI/10% foetal calf serum.

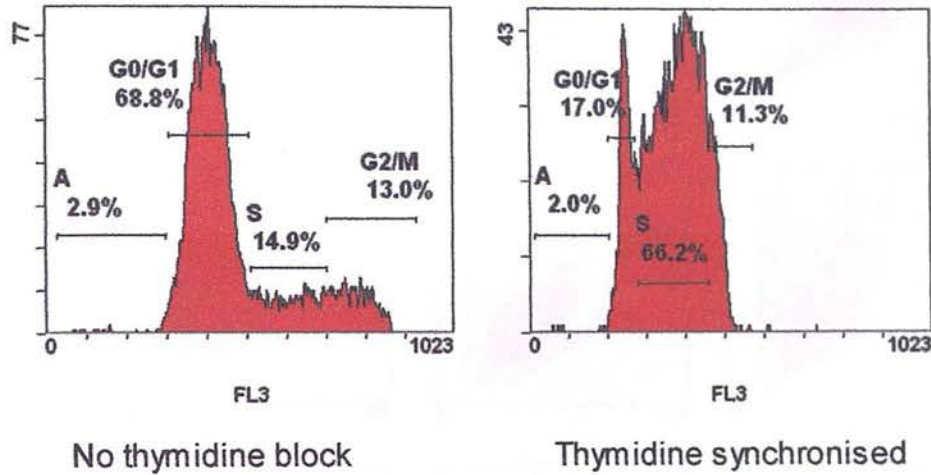
When the S-phase synchronised THP-1 cells were treated with 3 μ M camptothecin, cell death was apparent within 4 hours. Increasing the incubation times resulted in proportional increases in cell death (figures 3.4 & 3.5). Microscopic examination revealed that the number of necrotic cells was also increased as estimated by the proportion of lysed cells in culture. The expression of BOB78 among the treated and

Figure 3.2 Percentage cell death in THP-1 cells treated with camptothecin. Human leukaemic THP-1 cells were treated with varying concentrations of camptothecin for different periods as indicated. The cells were gated on morphology (dying in gate A and viable in V) and cell death correlated with reduced cell size as analysed on flow cytometry.



Rate of cell killing is relatively constant when the concentration of camptothecin is above 3μM. When the concentration of camptothecin is above 3μM the percentage of cell death (gated A) does not increase, indicating the rate of killing depends on the proportion of cells entering the DNA synthesis period (S-phase) of the cell cycle. The dose range selected appears to have lied on the plateau of a dose-response curve.

Figure 3.3 Synchronisation of leukaemic THP-1 cells using thymidine block. THP-1 cells were cultured and blocked in S-phase by incubation in 2mM thymidine for 18 hours. The cells were released from the block with washing and returned to standard culture. Cells were washed, permeabilised and stained with propidium iodide (read on FL3) to quantify genomic DNA.



Thymidine block increases proportion of cells in S-phase of cell cycle. The DNA quantity plots show about 60% of THP-1 cells to be arrested in S-phase of cell cycle. Apoptosis, reflected by the sub-G1 peak (D) resulting from leakage of degraded genomic DNA, is not increased by treatment with thymidine.

Within the cell cycle, **G1/G0 phase**: diploid state; **S phase**, synthesis of DNA; **G2/M phase**: 2 sets of diploid DNA; **A (or sub-G1) peak**: result of DNA degradation during apoptosis.

Figure 3.4 Cell death induced by camptothecin (CPT) treatment: a typical experimental result. Human leukaemic THP-1 cells were gated by size on flow cytometry. Periods of incubation with 3 μ M CPT listed. Increasing the period of incubation resulted in higher proportion of cells killed; cells in gate V were viable and cells gated in A were apoptotic.

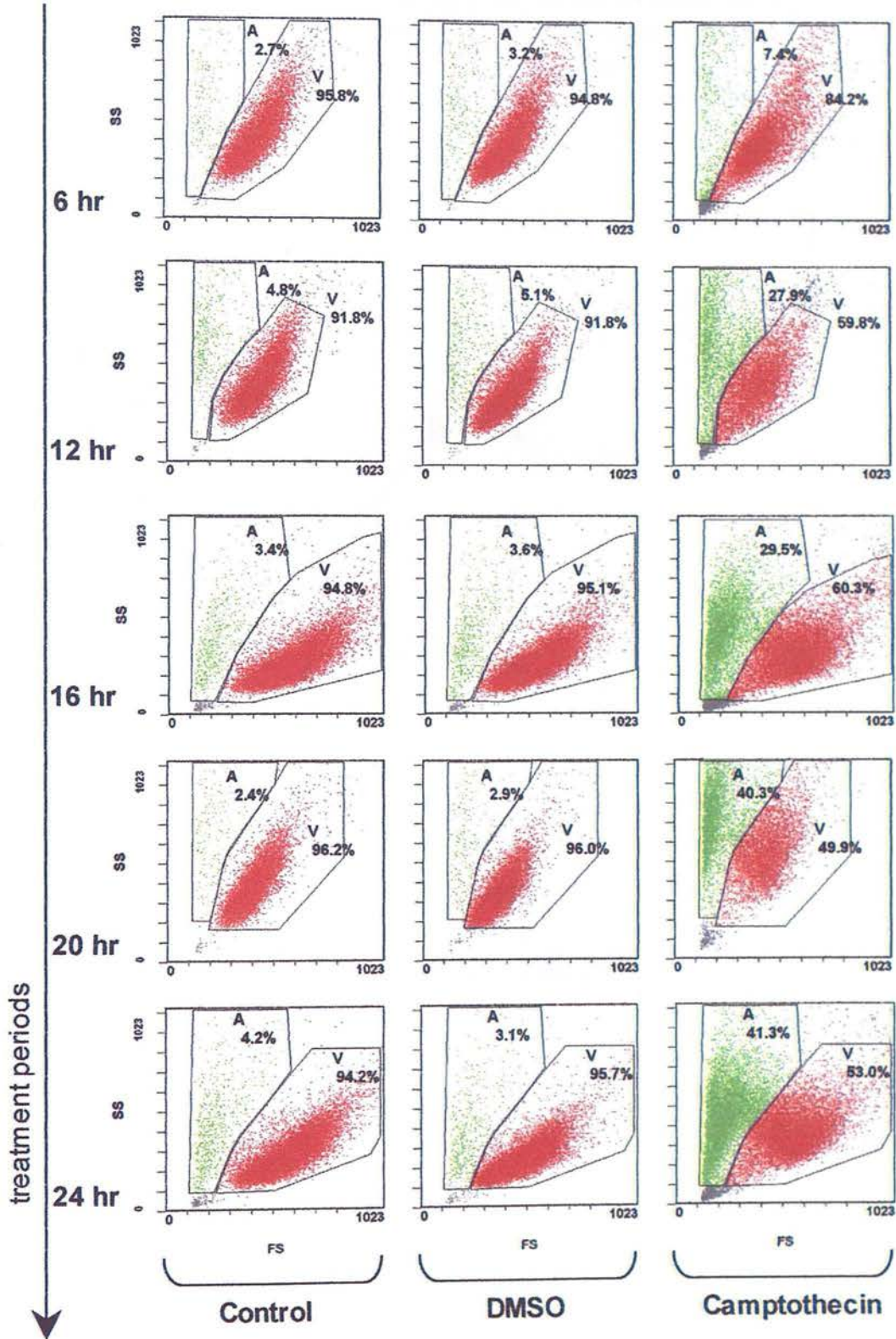


Figure 3.5 Cell death induced by camptothecin increases with period of incubation. Human leukaemic THP-1 cells were treated with 3 μ M camptothecin for 6, 12, 18 and 24 hours and sorted by morphology on flow cytometry, as shown in figure 3.4. The mean percentages of dying cells from replicate experiments were calculated from cells in gate A. Error bars represent standard errors calculated from the means of 3 experiments.

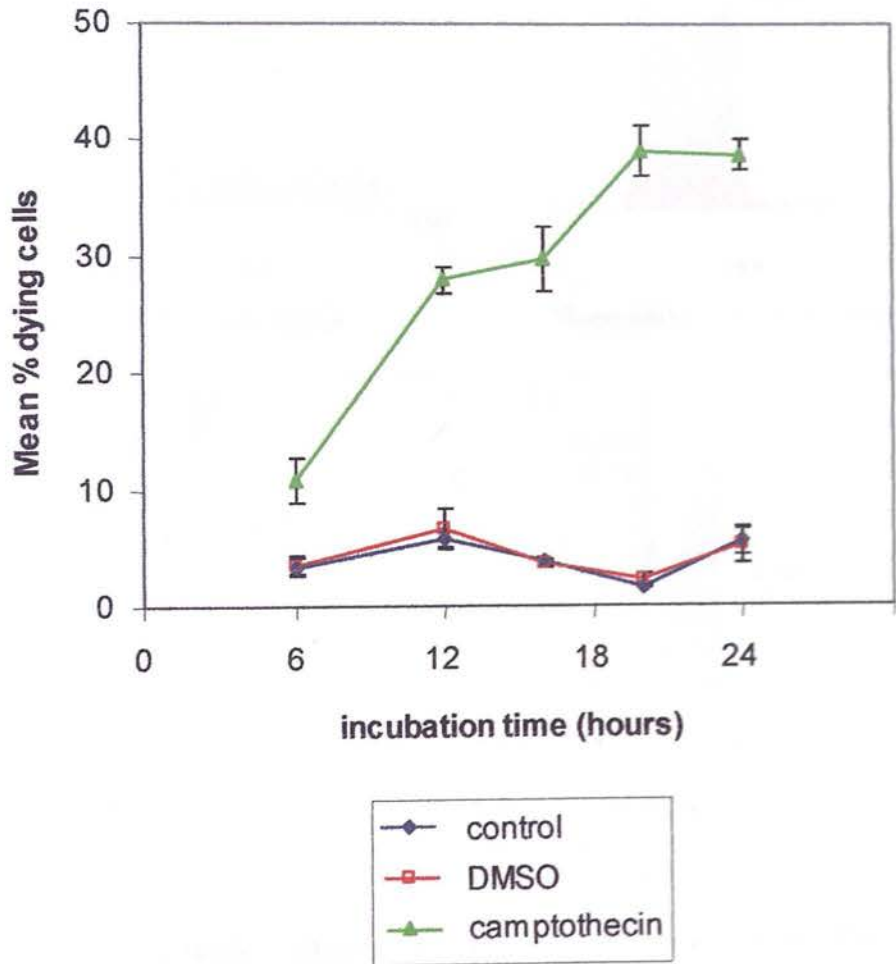
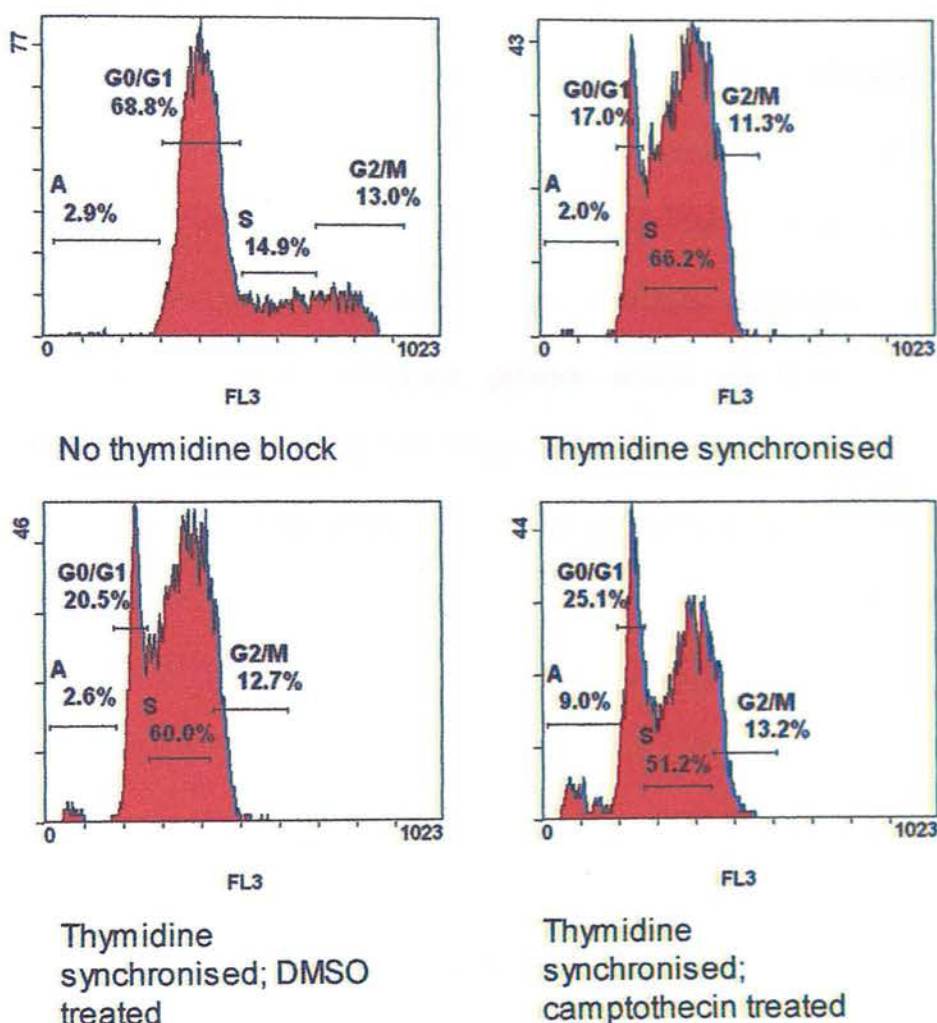


Figure 3.6 Degradation of cellular DNA in camptothecin treated THP-1 cells. THP-1 cells were cultured and blocked in S-phase by incubation in 2mM thymidine for 18 hours. The cells were released from the block with washing and returned to standard culture or incubated with 3 μ M camptothecin for 4 hours. The solvent DMSO was added as vehicle control. Cells were washed, permeabilised and stained with propidium iodide (read on FL3) to quantify cellular DNA.



Camptothecin induces apoptosis in THP-1 cells. The DNA quantity plots show about 60% of THP-1 cells to be arrested in S-phase of cell cycle. Camptothecin acts on susceptible cells in this phase. Apoptosis, reflected by the sub-G1 peak resulting from leakage of degraded genomic DNA, is increased compared with standard and DMSO conditioned cultures. Within the cell cycle, E: G1 phase with diploid state; C: S phase, DNA synthesized; B: M phase, 2 sets of diploid DNA; D: sub-G1 peak, result of DNA degradation.

untreated THP-1 cells was characterised through flow cytometry. Analysis of DNA content showed the typical sub-G1 peak indicating a subpopulation of cells undergoing apoptosis (figure 3.6).

Method 2. Induction of apoptosis through treatment with serum deprivation

The other method used for the induction of apoptosis was serum deprivation. This is a common method and probably results in apoptosis via mechanisms akin to withdrawal of cytokines, as serum contains many putative growth factors (Chao et al., 2002). Apoptotic cells were detected within 2 hours of serum deprivation. These cells exhibited propidium iodide exclusion profiles similar to those treated with camptothecin (see figures 3.8 and 3.9). When analysed for DNA content the apoptotic THP-1 cells showed up as the typical sub-G1 peak as before (figure 3.7). As discussed below, apoptotic cells staining weakly for propidium iodide showed strong surface expression of BOB78 antigen. The results suggest BOB78 antigen appears on the external cell membrane when a cell is committed to apoptosis. BOB78 antigen may therefore be a marker of apoptotic cells.

3.2.1 Apoptotic THP-1 cells express BOB78 on the external cell membrane

Human leukaemic THP-1 cells were induced to undergo apoptosis as described briefly above and in Methods (chapter 2). Cell death was documented with propidium iodide (PI) as a vital dye where nuclear staining is indicative of necrosis/very late apoptosis and propidium dim cells are indicative of apoptosis (Nicoletti et al., 1991; Del Bino, 1999). The cells were analysed by flow cytometry for the expression of BOB78

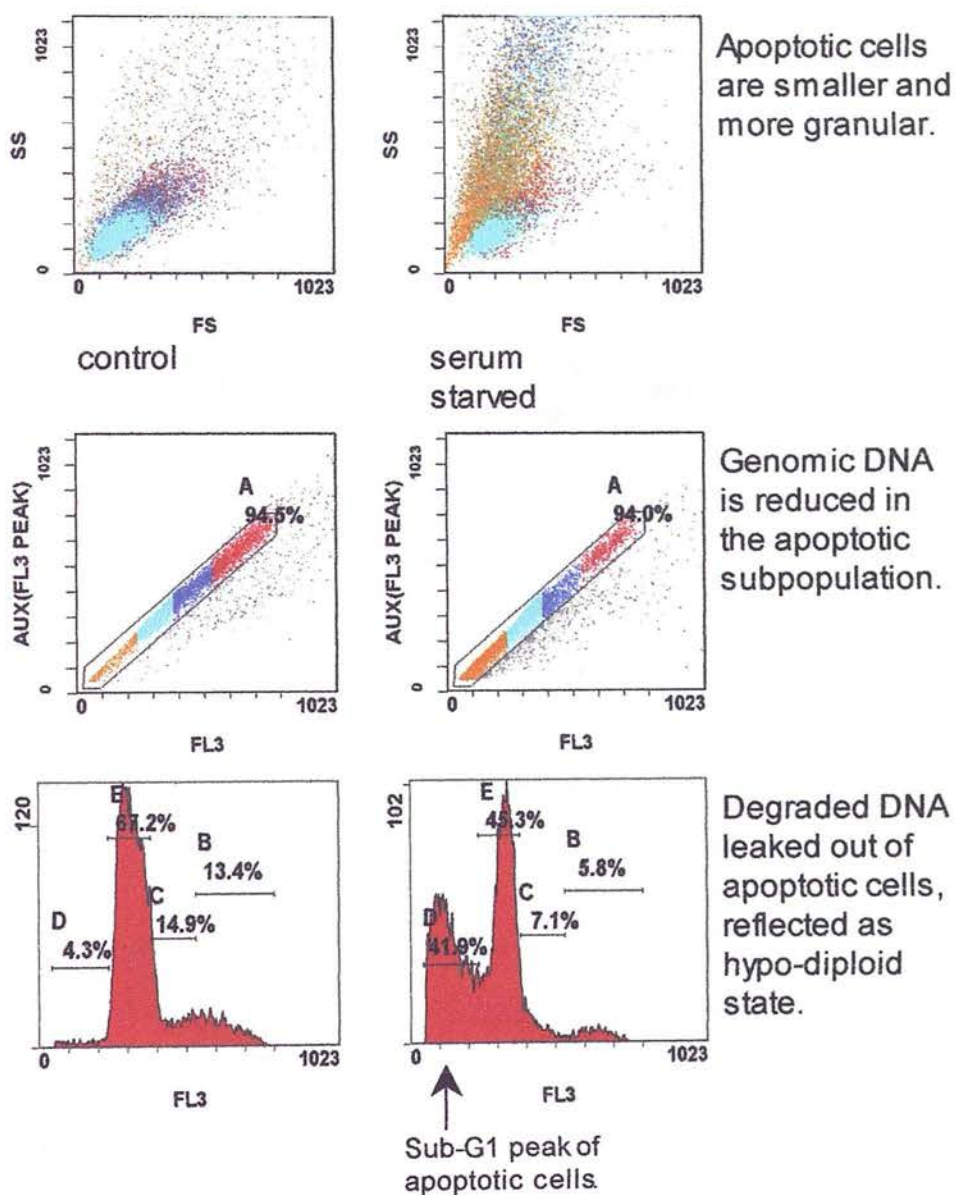
antigen. The results obtained by treatment with serum deprivation and camptothecin were similar (figures 3.8 and 3.9 respectively).

Four subpopulations of cells with different staining characteristics for PI and BOB78 were discernible. Viable cells did not stain for BOB78 antigen on the surface and excluded PI completely. Two subpopulations stained strongly for BOB78 antigen. One stained weakly for PI (PI dim) whilst the other stained strongly for PI (PI bright). The BOB78 antigen-positive/PI dim cells represent probably cells in early apoptosis. The BOB78 antigen-positive/PI bright cells would probably be in late apoptosis and losing membrane permeability. Lastly a population of PI bright cells were negative for BOB78 antigen staining. These were probably necrotic cells which were undergoing lysis. The apoptotic and necrotic cells fell into the same subpopulation based on size separation. It was not possible through gating to completely separate them into distinct populations. Shorter periods of camptothecin treatment or serum starvation resulted in more PI dim apoptotic cells, whilst longer treatment periods resulted in a greater collection of late apoptotic or necrotic cells. The observations from flow cytometry analysis suggest the BOB78 antigen appears on the plasma membrane when a cell undergoes apoptosis. Viable cells do not express BOB78 antigen on the surface at all. Necrotic cells which lose membrane integrity do not stain for BOB78 antigen on the surface.

3.3 Correlating expression of BOB78 to key events in apoptosis by flow cytometry

The specific appearance of BOB78 antigen on the surface coincided with two well characterized changes in apoptotic cells (Castedo et al., 1996; Vermes et al., 2000).

Figure 3.7 Degradation of cellular DNA in serum deprived THP-1 cells. THP-1 cells were synchronised through 18-hour incubation in 2mM thymidine. The cells were released from the thymidine block by washing and returned to culture in serum free RPMI for 16 hours. Cells in the control population had also been synchronised and released in the same manner. The cells were permeabilised and stained with propidium iodide (read on FL3) to quantify cellular DNA.



E: G1/G0 phase with diploid state

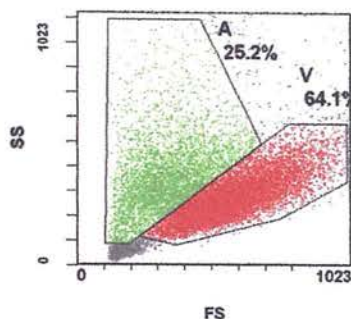
C: S phase, DNA synthesized

B: G2/M phase, 2 sets of diploid DNA

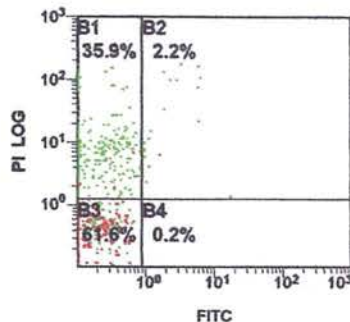
D: sub-G1 peak, result of DNA degradation



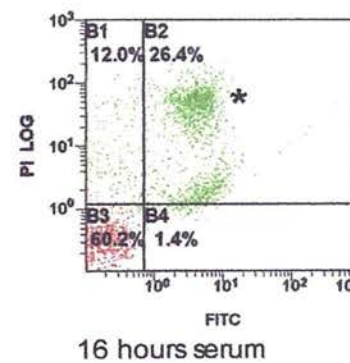
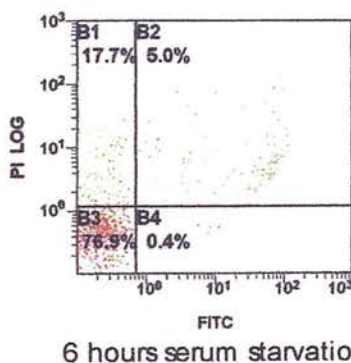
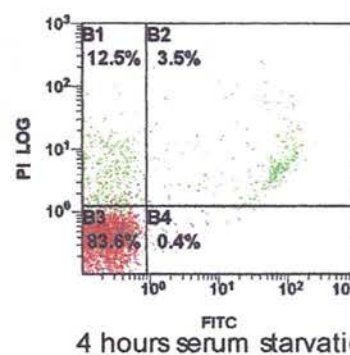
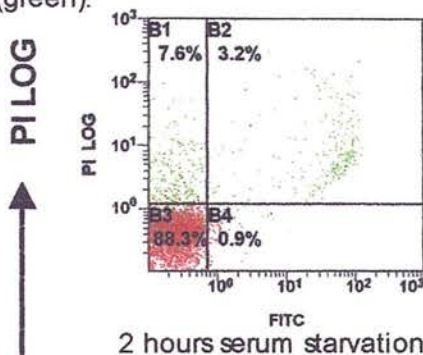
Figure 3.8 Staining THP-1 cells for surface expression of BOB78 antigen after serum starvation. Human leukaemic THP-1 cells were cultured in serum free medium for periods of 2, 4, 6 and 16 hours. The cells were stained for BOB78 antigen expression and isotype control IgM, with secondary detection by FITC-conjugated anti-mouse antibodies. Viability was assessed with propidium iodide (PI log) as analysed on flow cytometry. The cells were gated by size.



Differentiation by size. Viable (red) cells are bigger than dying cells (green).

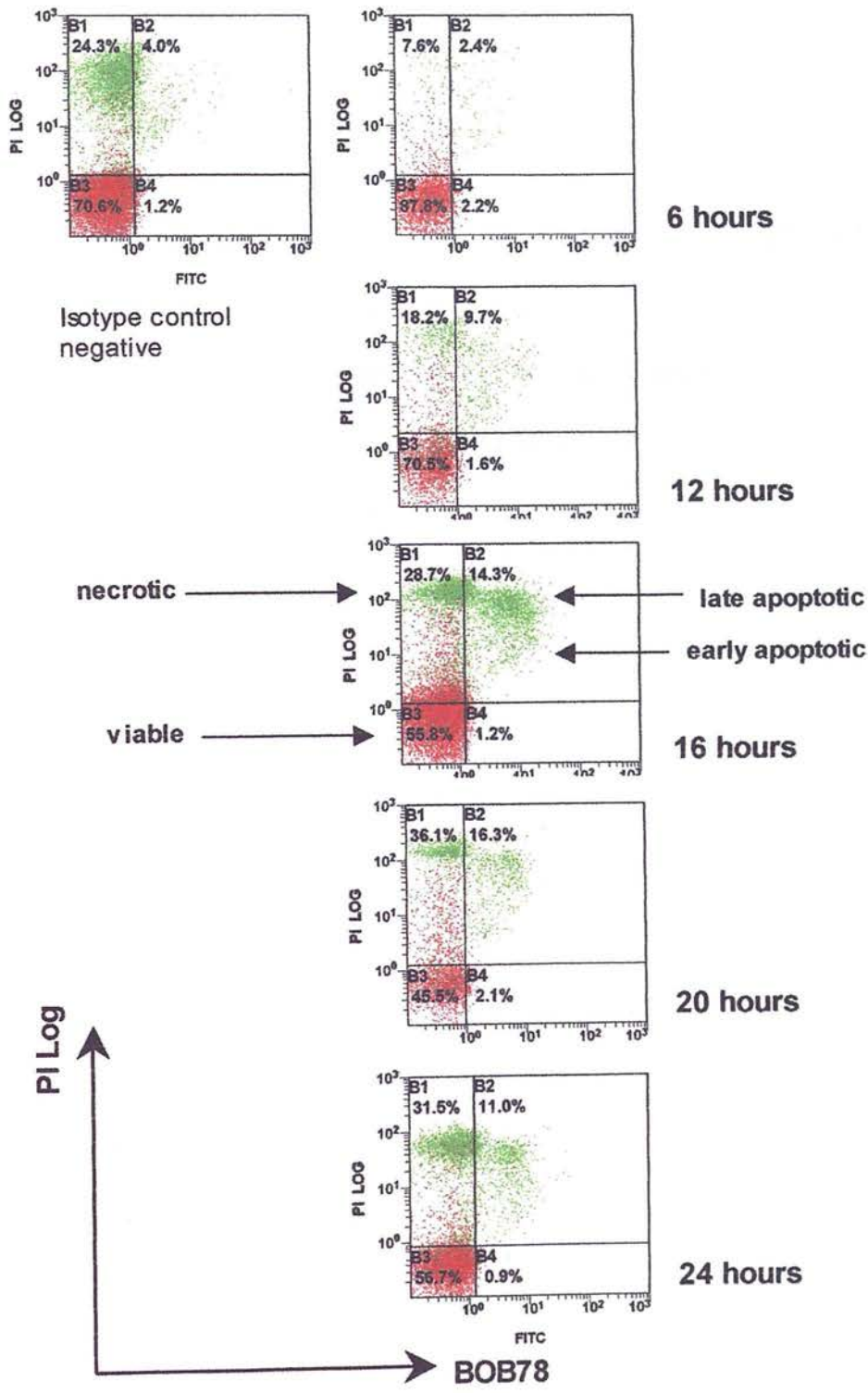


Control negative: THP-1 cells stained with IgM isotype control negative.



Apoptotic cells express BOB78 antigen on the surface. Viable cell (in gate V) did not stain BOB78 on the surface at all. Apoptotic cells (in gate A) with weak PI staining showed strong surface expression of BOB78. This expression was also evident in PI bright cells* which would represent late apoptotic cells prior to membrane lysis.

Figure 3.9 Staining THP-1 cells for surface expression of BOB78 after treatment with camptothecin. THP-1 cells were treated with 3 μ M camptothecin for periods of 6, 12, 16, 20 and 24 hours. Cells were stained for BOB78 antigen surface expression, detected with FITC-conjugated secondary antibody and analysed on flow cytometry. Composite dot-plots of the total populations on propidium iodide (red) and FITC (green) fluorescences. Dying THP-1 cells showed surface staining for BOB78 from early to late apoptosis. This surface feature is lost upon secondary necrosis.

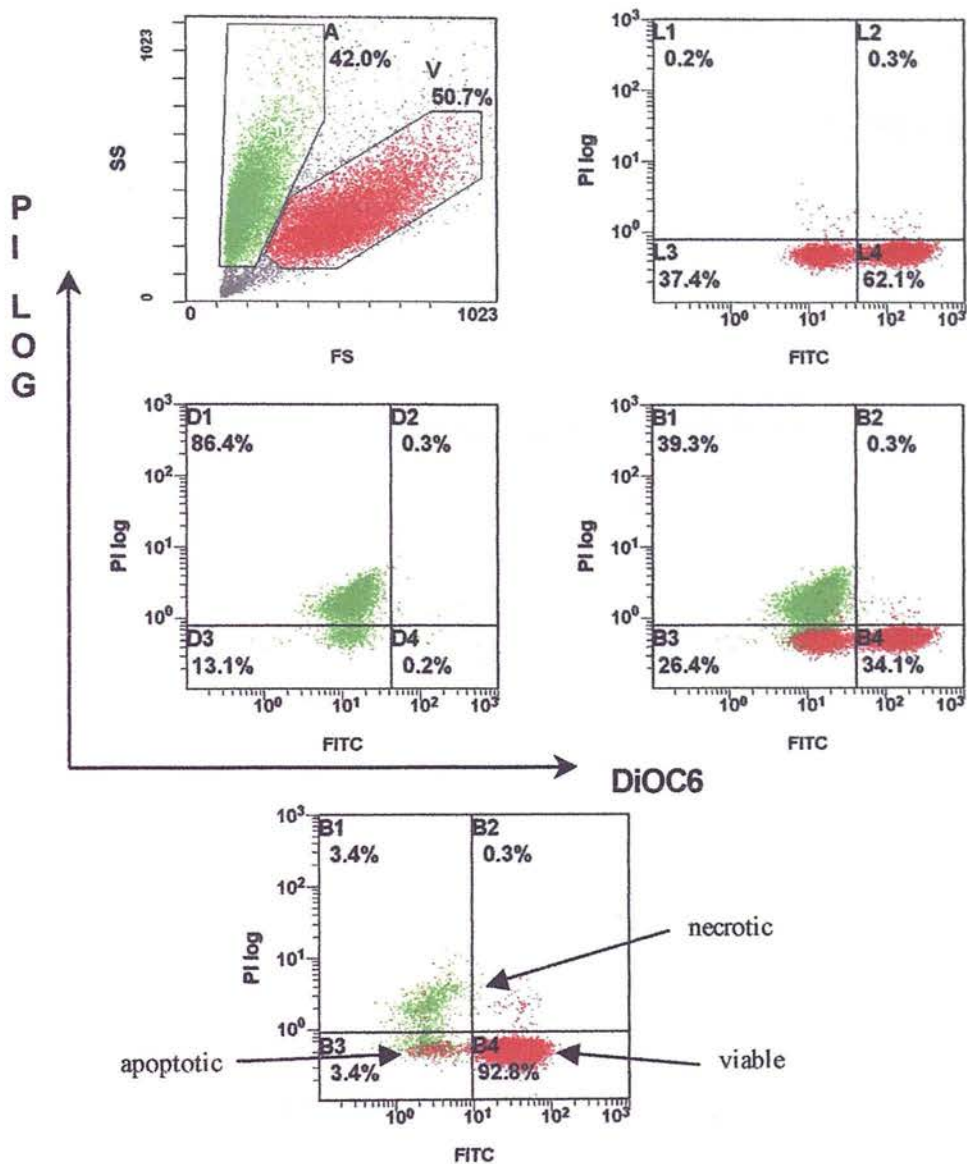


These were i) depolarisation of mitochondrial transmembrane potential (MTP), and ii) appearance of phosphatidylserine (PS) on the cell surface. Loss of MTP is an early event in cells committed to apoptosis (Crompton, 1999). The externalisation of PS residues onto the cell surface, detected through binding of Annexin-V, is another universal feature of apoptotic cells (Martin et al., 1995; van Engeland et al., 1998). The membrane expression of BOB78 antigen in apoptotic cells was tracked with these key events through dual staining and analysis by flow cytometry.

Method 1: Dynamic tracking of dying cells through loss of mitochondrial transmembrane potential (MTP)

When a cell loses the permeability barriers on the mitochondrial membranes, cytochrome-c is released and is incorporated into the complex of the apoptosome. This activates the initiator caspase, procaspase-9. This initiation phase of apoptosis is reflected by reduced staining for the dye DiOC6 (Overbeeke et al., 1998). This lipophilic cationic fluorochrome (green) is actively sequestered in the intermembranous space of mitochondria, and viable cells show strong uptake and maintain fluorescence when stained with this dye. Fluorescence is diminished when mitochondrial membranes lose their selective permeability and become leaky. This differential staining for DiOC6 would therefore label viable cells distinctly from dying cells. Within the population of dying cells, apoptotic ones are differentiated from the necrotic ones through the strong nuclear staining for propidium iodide (PI) by the latter. These characteristics were confirmed with camptothecin treated leukaemic THP-1 cells. Dose titration revealed 30mM DiOC6 was clearest in separating the various populations (figure 3.10).

Figure 3.10 Differentiation of viability by dual staining with DiOC6 and PI. THP-1 cells synchronised with 2mM thymidine for 18 hours and then treated with 3 μ M camptothecin for 6 hours. Mitochondrial fluorochrome DiOC6 (green) was added at room temperature to 30mM and reacted for 30 minutes. Propidium iodide (PI) was added immediately before analysis with flow cytometry. Viable cells were gated in V, and apoptotic cells in A.



Composite dot-plot from vehicle control showing typical dual staining characteristics.

Differentiation of cell viability. Viable cells maintain green fluorescence from sequestered DiOC6 (FITC) and exclude PI. Apoptotic cells demonstrate leakage of DiOC6 but exclude PI. Necrotic cells which have lost DiOC6 also stain with PI completely.

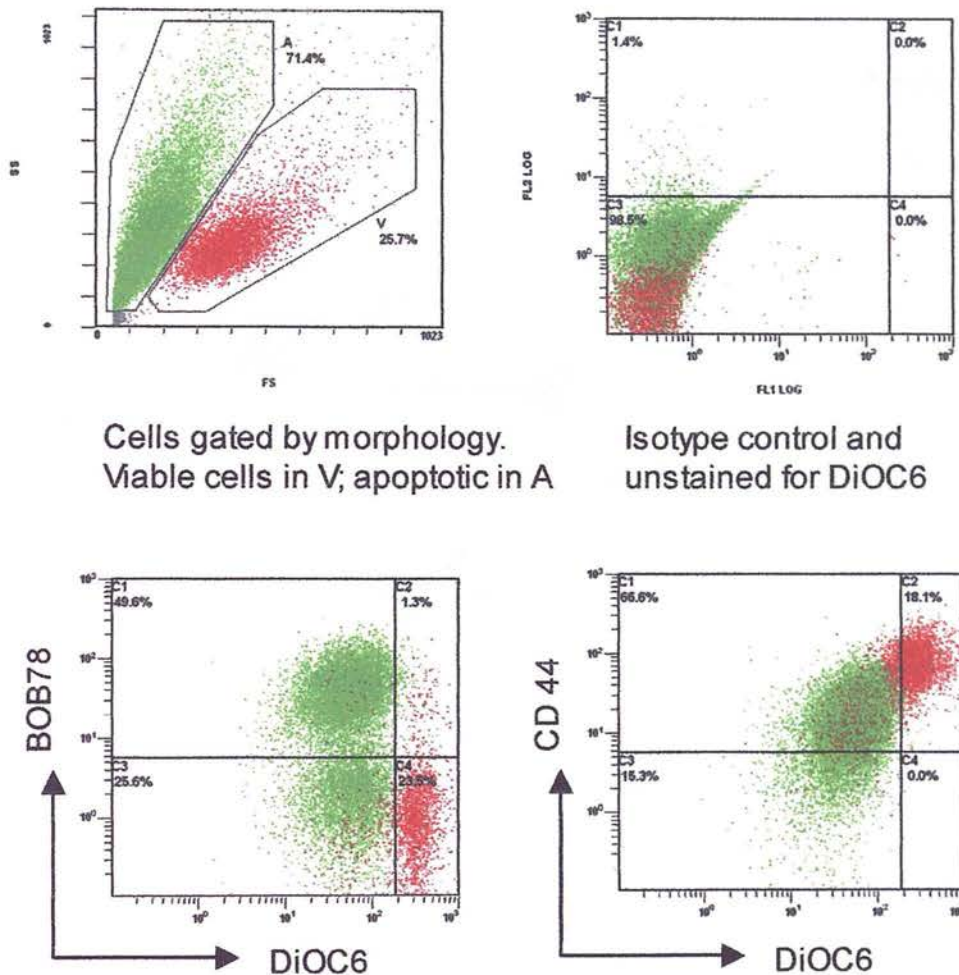
3.3.1 Cells which lose MTP show surface expression of BOB78 antigen

Human leukaemic THP-1 cells were induced to undergo apoptosis with 6 hours of camptothecin treatment in standard conditions as described earlier. Besides dual staining for BOB78 and PI, the same populations were also stained for BOB78 and DiOC6, and analysed by flow cytometry. Dying cells which shrank were gated according to size as reflected by reduced forward angle scatter (FS). Three subpopulations of cells were clearly distinguishable (figure 3.11). Viable cells stained strongly for DiOC6 and maintained the green fluorescence. These cells did not express BOB78 antigen on the surface. Another subpopulation which had lost MTP show marked surface expression of BOB78 antigen. A third subpopulation which was also dim in DiOC6 fluorescence did not express membrane BOB78 antigen. The cells which expressed BOB78 antigen on the surface also demonstrated two important characteristics. The cells were PI-dim and DiOC6-dim. It may be inferred that PI-dim cells which expressed surface BOB78 antigen had initiated apoptosis through depolarisation of MTP (figure 3.12). The DiOC6-dim population which did not stain for BOB78 antigen would represent late apoptotic or necrotic cells with membrane integrity being lost. These results indicate that cells in which irreversible initiation of apoptosis had occurred express BOB78 antigen on the surface.

Method 2: Correlating exposure of phosphatidylserine (PS) residues with BOB78 antigen expression by staining with Annexin-V

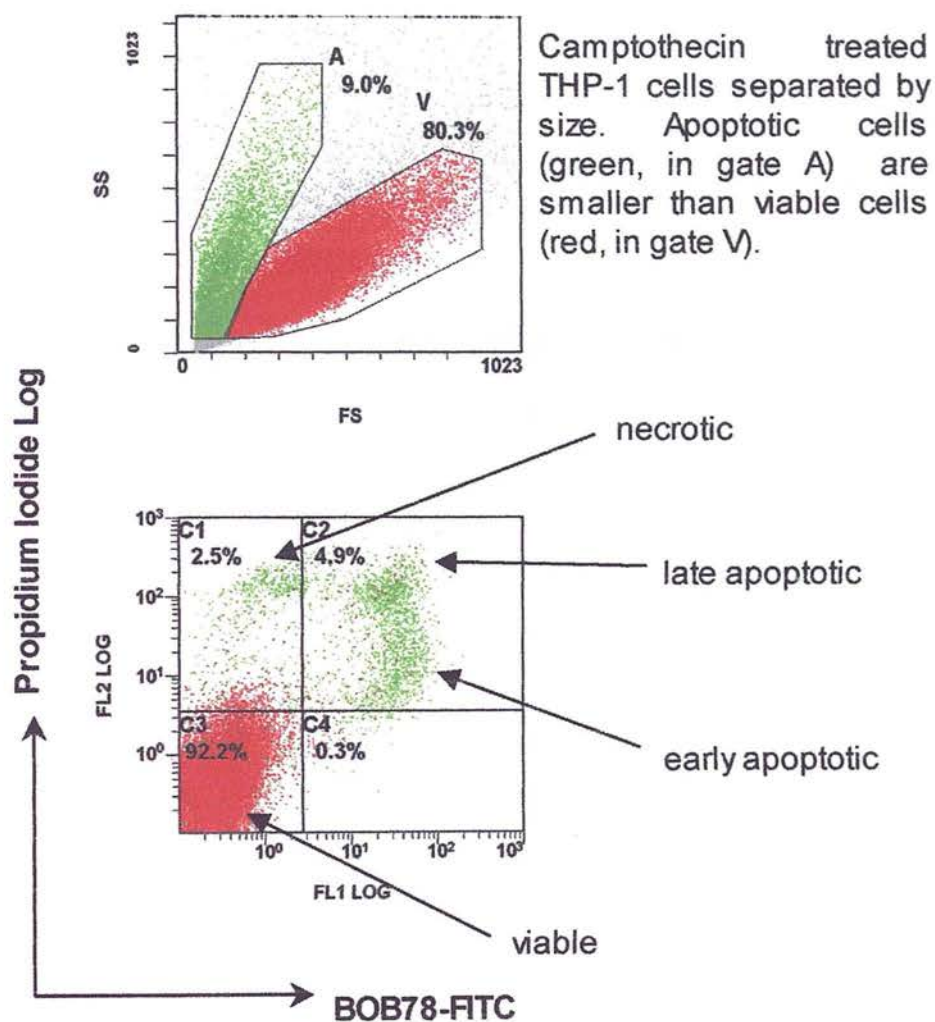
Presently, one of the best accepted features of apoptotic cells is their surface staining for Annexin-V (Martin et al., 1999; Martinez and Freyssinet, 2001). This soluble lipoprotein exhibits particular affinity for the PS residues displayed on the surface of apoptotic cells. In viable cells PS is restricted to the inner leaflet of the plasma

Figure 3.11 Expression of BOB78 antigen on DiOC6 dim cells. Human leukaemic THP-1 cells were treated with 3 μ M camptothecin for 6 hours and analysed with flow cytometry, for surface expression of BOB78 antigen, or CD44 (detected with anti-mouse PE-conjugated secondary antibodies) and integrity of mitochondrial transmembrane potential (maintenance of DiOC6 green fluorescence).



Surface expression of BOB78 antigen follows loss of the mitochondrial transmembrane potential (DiOC6 dim). A significant proportion of cells which have lost the mitochondrial transmembrane potential (MTP), reflected by the reduction in green DiOC6 fluorescence, express BOB78 antigen on the surface. The DiOC6 dim cells also lose the expression of surface adhesion molecule CD44. Surface expression of BOB78 antigen is an event which follows loss of MTP, a key event in the initiation of apoptosis.

Figure 3.12 BOB78 antigen expression correlated with staining for propidium iodide. Human leukaemic THP-1 cells were treated with 3 μ M camptothecin for 6 hours and analysed with flow cytometry. The cells were stained with monoclonal BOB78 (detected with anti-mouse FITC) and propidium iodide (PI) to assess membrane permeability.



Apoptotic cells express BOB78 antigen on the surface. Dual staining with BOB78 and PI. Viable cells exclude PI and do not express BOB78 antigen. Apoptotic cells, early or late, express BOB78. Necrotic cells lose BOB78 antigen expression on membrane lysis.

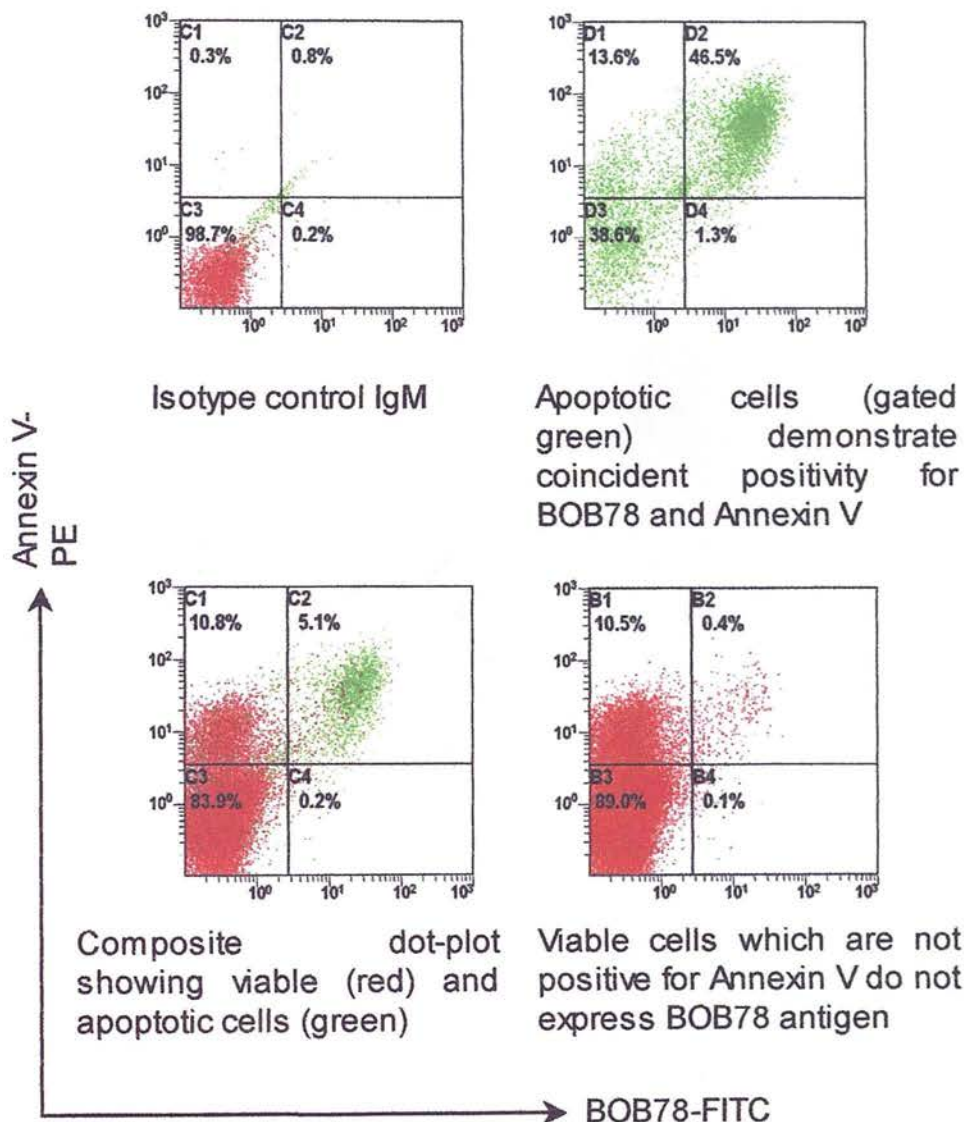
membrane (Devaux et al., 1991; Zachowski, 1993). There is movement of PS either way but enzymes called translocases maintain this asymmetric distribution (Diaz & Schroit, 1996). Externalisation of PS on apoptotic cells derives from a reversal of this traffic and is probably effected by the enzyme scramblase. In the presence of calcium ions, exposed PS binds strongly to Annexin-V. The same populations of cells from the experiments above stained for PI, BOB78 and DiOC6 were also examined for Annexin-V expression.

3.3.2 Cells which externalise phosphatidylserine (PS) also express BOB78 on the surface

Viable cells did not stain for BOB78 antigen or Annexin-V, and excluded PI completely. Within the population of dying cells surface staining for BOB78 antigen paralleled that of Annexin-V (figures 3.13 & 3.14). Those which expressed BOB78 antigen also stained for Annexin-V ie. expressed PS residues. Others which did not stain for Annexin-V also did not express surface BOB78 antigen. These probably represented cells which either had not yet exposed PS on the surface, or which had lost membrane integrity ie. undergone necrosis. This expression profile of BOB78 antigen indicates that surface expression of BOB78 antigen keeps pace with that of PS. Externalisation of PS, denoted by Annexin-V binding, had been suggested to be an early phenomenon in apoptotic cells. It precedes nuclear condensation and morphological changes. This implies the surface expression of BOB78 antigen may be an early feature in most apoptotic cells (Vermes et al., 1995; Martin et al., 1995)

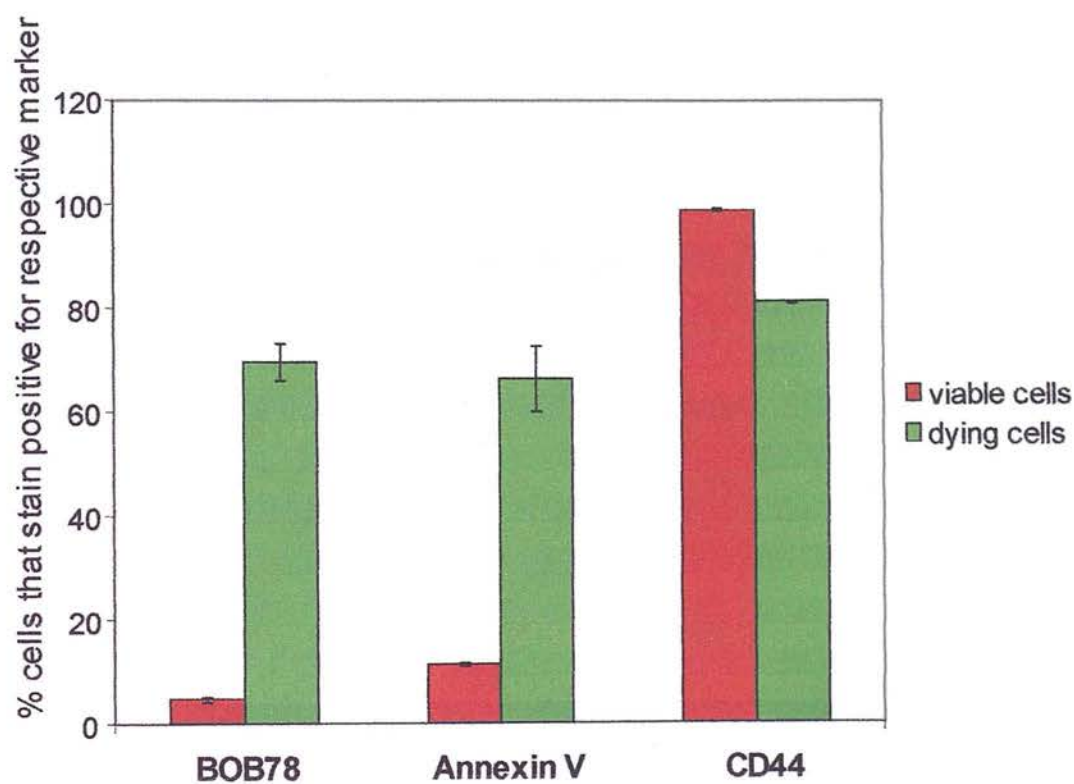
The findings obtained from flow cytometry suggest that expression of BOB78 antigen is not a terminal event before the cell disintegrates. Rather, once a cell proceeds with

Figure 3.13 BOB78 antigen expression coincident with positive staining for Annexin V. Human leukaemic THP-1 cells were treated with $3\mu\text{M}$ camptothecin for 6 hours and analysed with flow cytometry. The cells were stained with monoclonal BOB78 (detected with anti-mouse FITC) and PE-conjugated Annexin V.



Apoptotic cells express BOB78 antigen and phosphatidylserine on the surface. The coincident detection of BOB78 antigen and phosphatidylserine PS (through Annexin V) suggests BOB78 antigen is expressed on the surface of plasma membrane early in apoptosis.

Figure 3.14 Surface characteristics of viable cells vs. apoptotic cells with respect to BOB78 antigen, Annexin V and CD44 staining. Cells were treated with 3 μ M camptothecin for 6 hours, stained for BOB78 antigen, phosphatidylserine (by Annexin V) or CD44 and analysed by flow cytometry (see figures 3.11 & 3.13). Percentages of cells in the viable population that were positive for BOB78 antigen, Annexin V and CD44 were compared to those in the apoptotic population. Cells were gated into viable and apoptotic populations based on morphology. Error bars represent standard errors, calculated from replicate experiments.



apoptosis BOB78 antigen appears on, or is translocated to, the surface, possibly as an early marker of cell death.

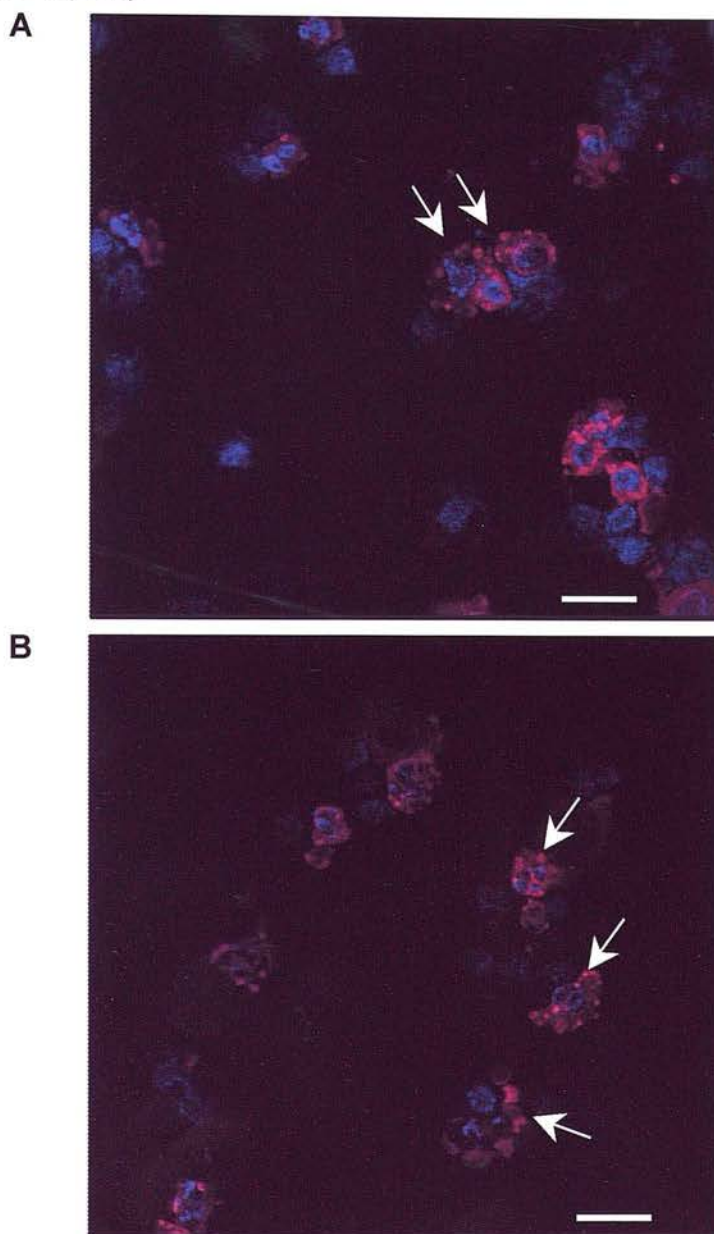
3.4 Mapping BOB78 antigen expression on apoptotic cells with fluorescence microscopy

The localisation of BOB78 antigen on apoptotic cells was also studied with fluorescence immunocytochemistry. Apoptosis was induced in THP-1 cells with 3 μ M camptothecin for 16 hours, after having been first synchronised in the S-phase of cell cycle through incubation in 2mM thymidine for 18 hours (Del Bino et al., 1991). Apoptotic cells were harvested following a low g centrifuge. These cells were then cytocentrifuged onto microscopic slides and processed for immunocytochemistry as described in Methods (chapter 2). The cells were stained with IgM control negative and monoclonal BOB78 antibodies. Nuclear changes identifying apoptosis were followed with TO-PRO 3 staining. The staining was visualised with confocal microscopy.

3.4.1 BOB78 localises to blebs of apoptotic cells

Analysis by confocal microscopy revealed BOB78 to be concentrated in the blebs of apoptotic cells (figure 3.15). In apoptotic THP-1 cells with typical nuclear fragmentation BOB78 antigen was disproportionately distributed to the blebs. This pattern of distribution was apparent even though the cytoplasm in apoptotic cells was condensed. Cells which contained condensed nuclei (probably prior to fragmentation) showed BOB78 antigen to be concentrated nearer the periphery of the cytoplasm. Necrotic cells were discernible from apoptotic ones. These necrotic cells had nuclei which did not exhibit typical apoptotic clumping (figure 3.16). The nuclei were relatively amorphous and larger, and there was little or no cytoplasm. There was no

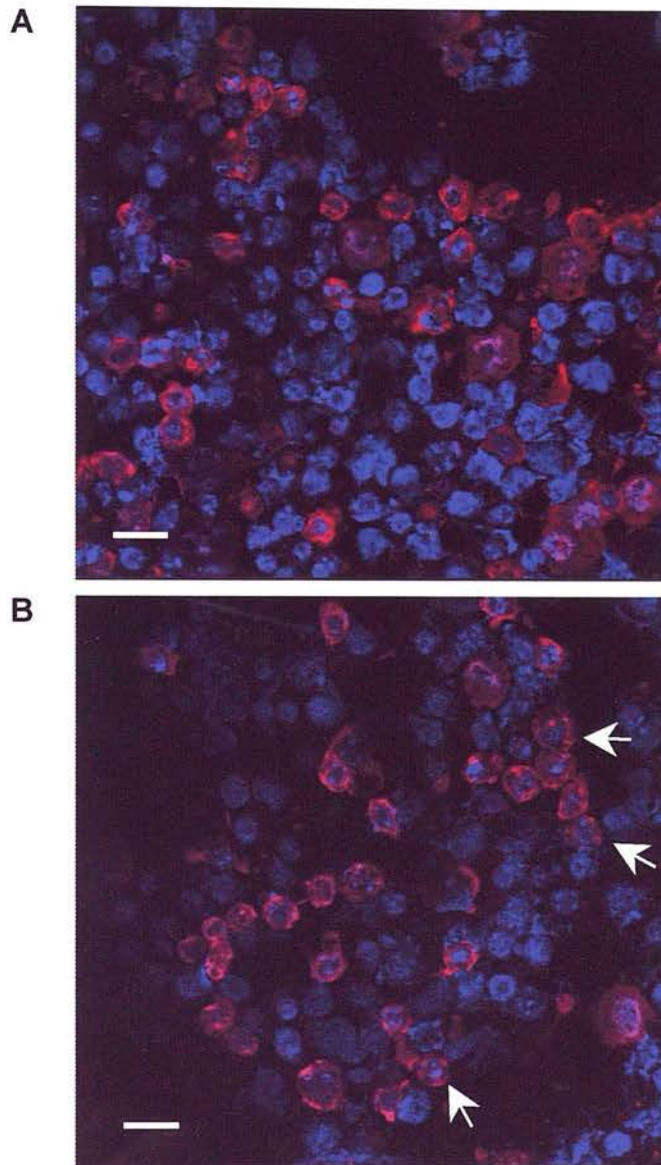
Figure 3.15 **Microscopic localisation of BOB78 antigen in the membranes of apoptotic THP-1 cells.** Human leukaemic THP-1 cells were treated with 3 μ M camptothecin for 16 hours. The THP-1 cells were harvested on and cytocentrifuged onto microscopic slides. Fluorescence immunocytochemistry was performed with monoclonal BOB78 and IgM isotype control antibody. Primary reaction was detected with fluorescent TRITC (red) conjugated secondary rabbit anti-mouse antibody. Nuclear staining was effected with TOPRO-3 (blue).



A: membrane blebs and BOB78. Apoptotic THP-1 cells with typical nuclear clumping appear to develop membrane blebbings (arrows). Those which are blebbing appear to have BOB78 antigen concentrated to the periphery of the cytoplasm or membranes. Naked nuclear materials probably result from lysis of late apoptotic or necrotic cells. (Bar: 15 μ m)

B: membrane blebs and BOB78. The blebs within apoptotic cells appear to concentrate BOB78 antigen (red) within them (top arrows). The staining pattern seen with the bottom-arrowed cell may be due to disintegration of the cell upon secondary necrosis. (Bar: 15 μ m)

Figure 3.16 Microscopic comparison of BOB78 antigen expression in apoptotic and necrotic THP-1 cells. Human leukaemic THP1 cells were treated with 3 μ M camptothecin for 16 hours. The cells were harvested and cytocentrifuged onto microscopic slides. Fluorescent immunocytochemistry was performed with monoclonal BOB78 and IgM isotype control antibody. Primary reaction was detected with fluorescent TRITC (red) conjugated secondary rabbit anti-mouse antibody. Nuclear staining was effected with TOPRO-3 (blue).



A: a mixture of apoptotic and necrotic cells. THP-1 cells with nuclear condensation and clumping typical of apoptosis show BOB78 antigen localising mainly to the periphery of the cytoplasm. These are distinguished from necrotic cells which appear as naked nuclei lacking typical apoptotic features. (Bar: 15 μ m)

B: membrane blebs and BOB78. Some apoptotic THP-1 cells with typical nuclear clumping appear to develop membrane blebblings (arrows). Those which are blebbing appear to have BOB78 antigen concentrated to the periphery of the cytoplasm or membranes. Naked nuclear materials probably result from lysis of late apoptotic or necrotic cells. (Bar: 15 μ m)

positive staining for BOB78 antigen in necrotic cells. The findings suggest expression of BOB78 antigen on the surface is maintained only on apoptotic cells. Where membrane integrity had been lost upon secondary necrosis, this identifiable feature of apoptotic cells disappears.

3.4.2 BOB78 antigen translocates from cytoplasm to membrane surface in apoptotic cells

The localisation of BOB78 antigen to membrane blebs of apoptotic cells was studied further in cells with abundant cytoplasm. The hepatoma-derived HUH-7 cells were treated with a toxic dose of monensin at 25 μ M for about 12 hours. Monensin interrupts protein traffic between Golgi and endoplasmic reticulum (Mollenhauer et al., 1990). It can be used to stress cells at low doses (in terms of protein traffic), and can cause death through apoptosis in susceptible cells (Park et al., 2002; Park et al., 2003). The BOB78 antigen was observed to outline blebs on the membrane of apoptotic cells, as well as blebs which had been released (figure 3.17). Neighbouring cells which were not blebbing showed BOB78 antigen distributed throughout the cytoplasm. This suggests that in apoptotic cells BOB78 antigen relocates from the cytoplasm to the membrane blebs. Closer scrutiny revealed that BOB78 antigen collected close to where the plasma membrane began to evaginate, and blebs that appeared ready for budding off were filled with BOB78 antigen. The few blebs which budded off into the vicinity of neighbouring cells were lined with BOB78 antigen.

This pattern of BOB78 antigen translocation was also observed when HUH-7 cells were treated with toxic doses of cycloheximide (figure 3.18). Cycloheximide interferes with protein biosynthesis. Membrane blebs accumulated BOB78 antigen whilst being

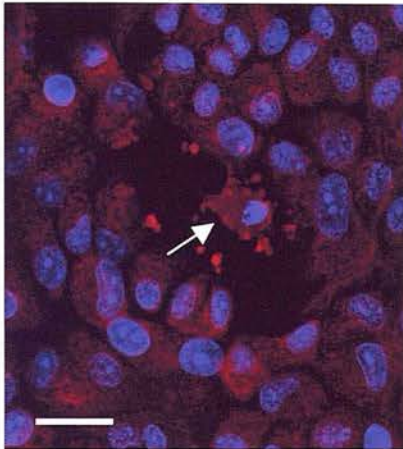
developed at the cell surface. Some BOB78-positive blebs were seen to ferry fragmented nuclear materials (figure 3.19). An incidental finding was that the cytoplasmic distribution of BOB78 antigen in those cells which did not undergo apoptosis did not differ from untreated cells (figure 3.18). This observation may suggest a low basal turnover of BOB78 antigen in the cells. A molecule which undergoes a higher turnover would show obvious disruption or deviation of immunostaining after treatment with cycloheximide. The main cytoplasmic pool of the BOB78 antigen may therefore be involved in this translocation to the cell surface.

DISCUSSION

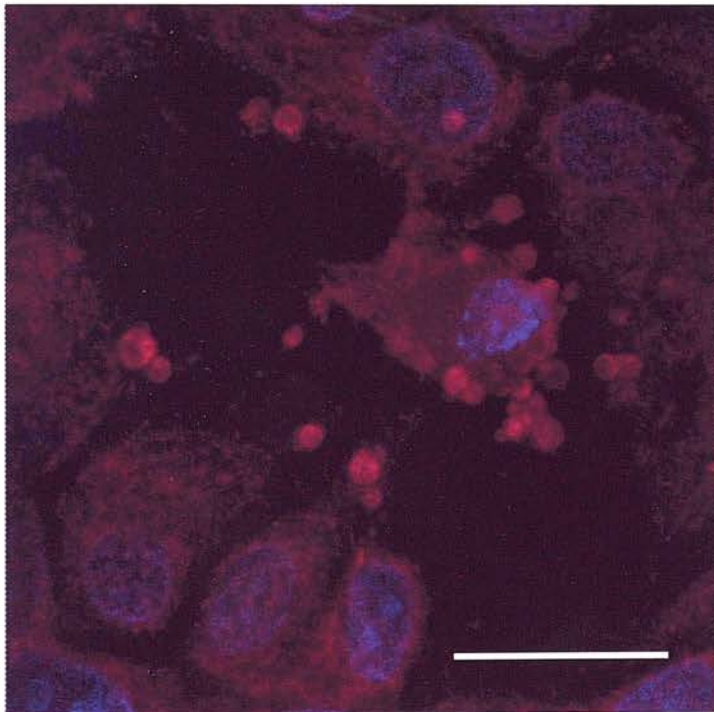
The BOB78 antigen is normally present within the cytoplasm of cells and appears at the plasma membrane during apoptosis. The surface expression of BOB78 antigen on cells appears to be consistent regardless of the mechanism of apoptosis induction. This ability of the BOB78 antibody to identify apoptotic cells was first characterised in neutrophils (Hart et al., 2000) where surface expression of BOB78 antigen appeared to be a late marker of apoptosis. There appears to be no specific lineage confinement of BOB78 antigen in nucleated cells. Since apoptosis is run on a highly conserved programme, BOB78 antigen may be intimately linked with cell death. The kinetics and role of the BOB78 antigen in normal cells may elucidate this possible association with the apoptotic process. These are elaborated in Chapter 4.

The surface staining of BOB78 detected with flow cytometry suggests that BOB78 antigen localises to membranes of cells undergoing apoptosis. Since apoptotic cells develop membrane blebs, BOB78 antigen may be localising to these evaginations at the cell surface. Analysis with confocal microscopy has confirmed this association. The

Figure 3.17 Localising BOB78 antigen in apoptotic HUH-7 hepatoma cells treated with monensin. Human hepatoma HUH-7 cells were grown in monolayer and treated with 25 μ M monensin for 16 hours. Immunocytochemistry was performed with monoclonal BOB78 and IgM isotype control antibodies. Reaction was detected with TRITC-conjugated (red) rabbit anti-mouse antibodies. Nuclear counter-staining was effected with TO-PRO 3 (blue).

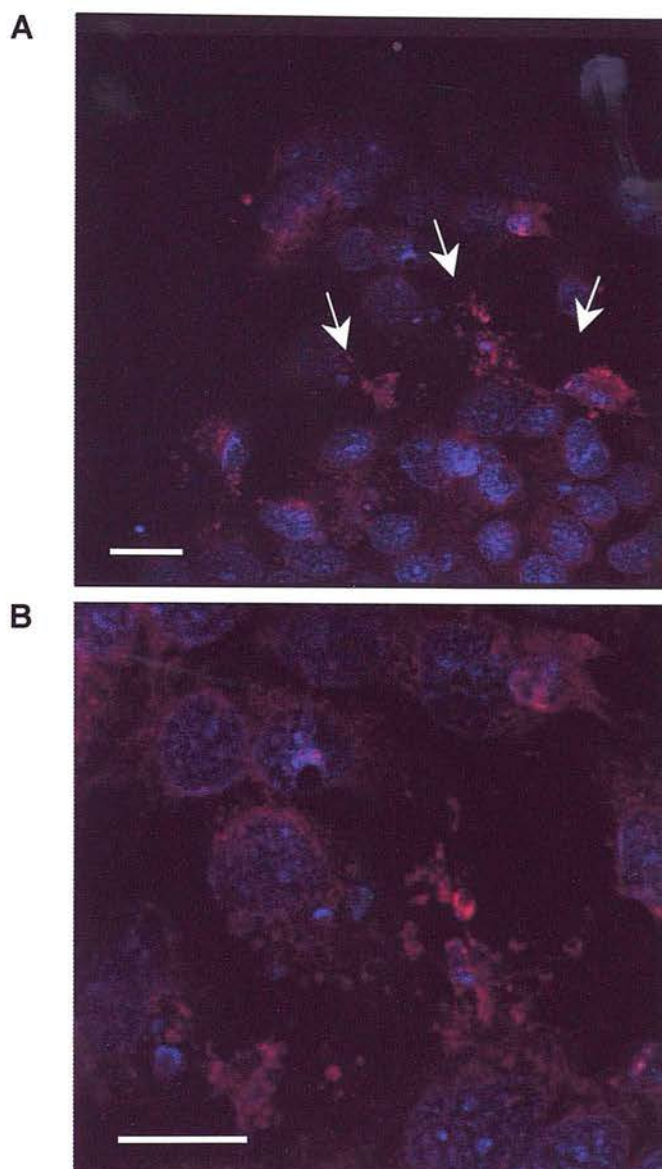


Frame 1: An apoptotic cell is seen within a monolayer of HUH-7 cells (arrowed). The apoptotic HUH-7 cell shows a condensed nucleus and blebs budding off the cytoplasm. The blebs show prominent BOB78 localisation. The nuclei within non-apoptotic cells appear larger and contain nucleoli. (Bar: 25 μ m)



Frame 2: magnified view of frame 1 focussing on the apoptotic cell. Blebs are seen to be lined with BOB78. At the cytoplasm where the membrane blebs develop, BOB78 appear to be channelled into these, and adhere to the membrane of the blebs as these bud off. (Bar: 25 μ m)

Figure 3.18 Microscopic characterisation of BOB78 antigen in apoptotic HUH-7 cells treated with cycloheximide. Human hepatoma HUH-7 cells were induced to undergo apoptosis with 10 μ g/ml cycloheximide for 6 hours. The cells were stained with primary monoclonal BOB78 and IgM isotype control. Reaction was detected with TRITC-conjugated (red) secondary rabbit anti-mouse antibodies and visualised using confocal microscopy.

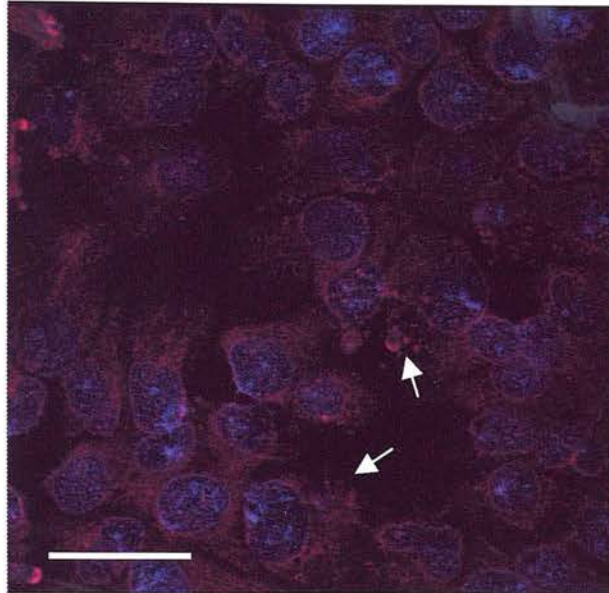


A: membrane blebs and BOB78. Apoptotic cells are identified with membrane blebbing. BOB78 antigen is seen to concentrate in the blebs. The nuclei in the apoptotic cells had fragmented and the clumps appear to be carried off in the blebs. Some cells showed prominent localisation of BOB78 antigen to the peripheral cytoplasm. These had nuclei which showed no clumping. These cells may represent early apoptotic ones. The streaming of BOB78 to the periphery and the concentration of it in the blebs in these cells would correlate with surface staining detected on flow cytometry. (Bar: 25 μ m)

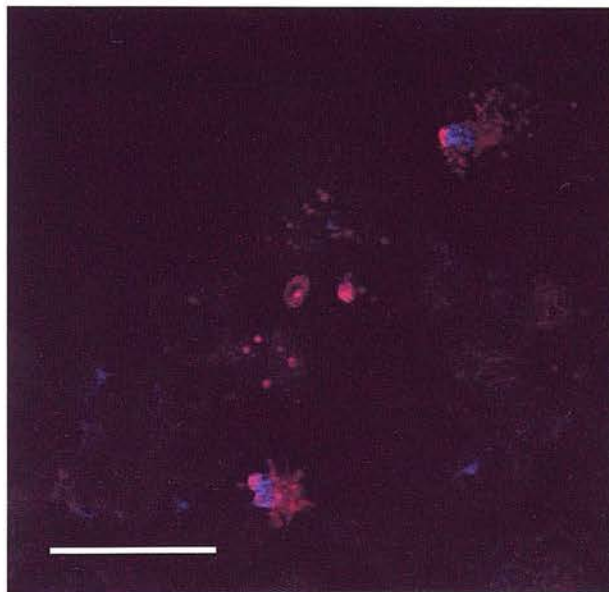
B: BOB78 antigen concentrated in blebs. Magnified view of A focussing on the blebbing cells. Concentration of BOB78 antigen to the blebs of the apoptotic cells can be seen. The fragmented nuclear materials appear to be contained within the blebs which are lined with BOB78 antigen. (Bar: 25 μ m)

Figure 3.19 Microscopic localisation of BOB78 antigen in apoptotic bodies. Human hepatoma HUH-7 cells were induced to undergo apoptosis with 10 $\mu\text{g/ml}$ cycloheximide for 6 hours. The cells were stained with primary monoclonal BOB78 and IgM isotype control. Reaction was detected with TRITC-conjugated (red) secondary rabbit anti-mouse antibodies and visualised using confocal microscopy. Frame 2 was imaged at a focal plane lying above that of frame 1.

Frame 1



Frame 2



Frame 1: Detaching apoptotic cell. Attached HUH-7 cells growing in monolayer. Some cells in apoptosis revealed blebbing of the membranes (arrows). The other cells with normal appearing nuclei showed typical cytoplasmic distribution of BOB78 antigen. (Bar: 25 μm)

Frame 2: Detaching apoptotic bodies. The blebs coming off the cells were imaged at a level above the focal plane in frame 1. BOB78 antigen can be seen localising to the blebs. Apoptotic bodies with fragmented nuclear materials detached from the monolayer and were lined with BOB78 antigen. (Bar: 25 μm)

apoptotic cells with BOB78 antigen localising to the periphery of the cytoplasm and membrane blebs would correspond with the smaller cells which were propidium iodide-dim and expressing BOB78 antigen on the surface. The bodies which stain strongly for propidium iodide but do not express BOB78 antigen would correspond with the naked nuclei seen on microscopy. These cells probably had undergone membrane lysis upon secondary necrosis. It appears that BOB78 antigen translocates from its normal cytoplasmic location to the plasma membrane and blebs during apoptosis. BOB78 antigen is concentrated in apoptotic blebs and remains confined to the membranes of apoptotic bodies as these are released during cellular re-assembly in cell death.

Treatment of human hepatoma HUH-7 cells with monensin or cycloheximide did not result in discernible changes of cytoplasmic BOB78 antigen expression. The global distribution of BOB78 within the cytoplasm of treated or untreated cells appeared to be similar. These observations suggest that the normal basal turnover of BOB78 antigen within cells is low. Since protein biosynthesis is generally halted during apoptosis (Hengartner, 2000), the strong expression of BOB78 antigen in the membranes and blebs indicate it is likely that BOB78 antigen is not actively synthesised during apoptosis. However, one may speculate that the movement of BOB78 antigen from cytoplasm to membrane may be an active process requiring energy input.

It remains to investigate what role BOB78 antigen may have in lining the membranes of apoptotic blebs. Whether this expression is part of the apoptosis machinery, part of normal cellular machinery mobilised to maintain cellular integrity during cellular reassembly (or disassembly), or is part of important recognition signals for macrophages remain to be investigated.

Closer scrutiny of the staining pattern of BOB78 on immunocytochemistry suggests that the BOB78 antigen may be potentially expressed in the nuclei of cells. The staining within the nuclei has not, however, been consistent and might have been due to variability in the fixation of the cells, in spite of the same protocol having been used for all experiments. Nevertheless, this possibility needs further study and verification. It is interesting to note that heat shock protein 70 (hsp70) has been found to be expressed in the nuclei of stressed cells (Ramage and Guy, 2004). It is likely that hsp70 may be influencing transcription within the nucleus under certain conditions. It is possible therefore that hsp60, being another molecular chaperone, may be involved in similar activity within the nucleus. The potential of hsp60 being intimately linked to transcriptional activity within stressed cells warrants further investigation.

CHAPTER 4

CHARACTERISATION OF THE BOB78 ANTIGEN WITHIN THE CELL

INTRODUCTION

The presence of BOB78 antigen in the human leukaemic THP-1 and hepatoma HUH-7 cell lines suggests this antigen might not be lineage limited in expression. These cells demonstrate widely varying behaviour with respect to motility and biosynthetic capability. The cytoplasmic localisation of BOB78 antigen within these cells suggests it might be an entity vital to fundamental cellular functions. At the same time the nature of BOB78 antibody being of IgM subclass also raises the possibility that it might be recognising a carbohydrate moiety. This, if proven true, would mean that the antibody may not be localising to any specific protein antigen, and that isolating the BOB78 antigen would be difficult. The abundant staining of BOB78 antigen within the cytoplasm of the cells studied so far prompted queries as to the location of BOB78 within the biosynthetic-secretory pathway. The appearance of BOB78 antigen at the plasma membrane during apoptosis suggests that the dynamics of this phenomenon may be linked to the endomembrane systems of the cell. This chapter details the studies performed in an attempt to further evaluate the nature of BOB78 antigen.

RESULTS

4.1 Verifying the lineage fidelity of BOB78 antigen

Observations with fluorescence microscopy of BOB78 antigen have already indicated that it is normally present in the cytoplasm of the leukaemic (THP-1) and hepatoma (HUH-7) cells studied (refer Chapter 3, parts 3.1 and 3.4). Normal viable cells do not express BOB78 antigen on the surface. The surface expression of BOB78 antigen on apoptotic cells appears to be due to the translocation of the molecule from the cytoplasm to the plasma membrane. The presence of BOB78 antigen was thus tested in various normal human cells and human cancer cell lines. In addition, in order to investigate if it might be a highly conserved antigen, BOB78 antigen expression was also examined in the mouse myeloma cell line Sp2/0. Analysis with flow cytometry was utilised to assess the cytoplasmic expression of the BOB78 antigen in these cells. Cells from various lineages, normal and cancer-derived, were cultured in standard culture conditions and harvested for immunostaining. The cells were fixed and permeabilised (Pollice et al., 1992), and stained with antibodies for analysis using flow cytometry as described in Materials & Methods (chapter 2).

4.1.1 BOB78 is highly conserved and is normally present in the cytoplasm of nucleated cells

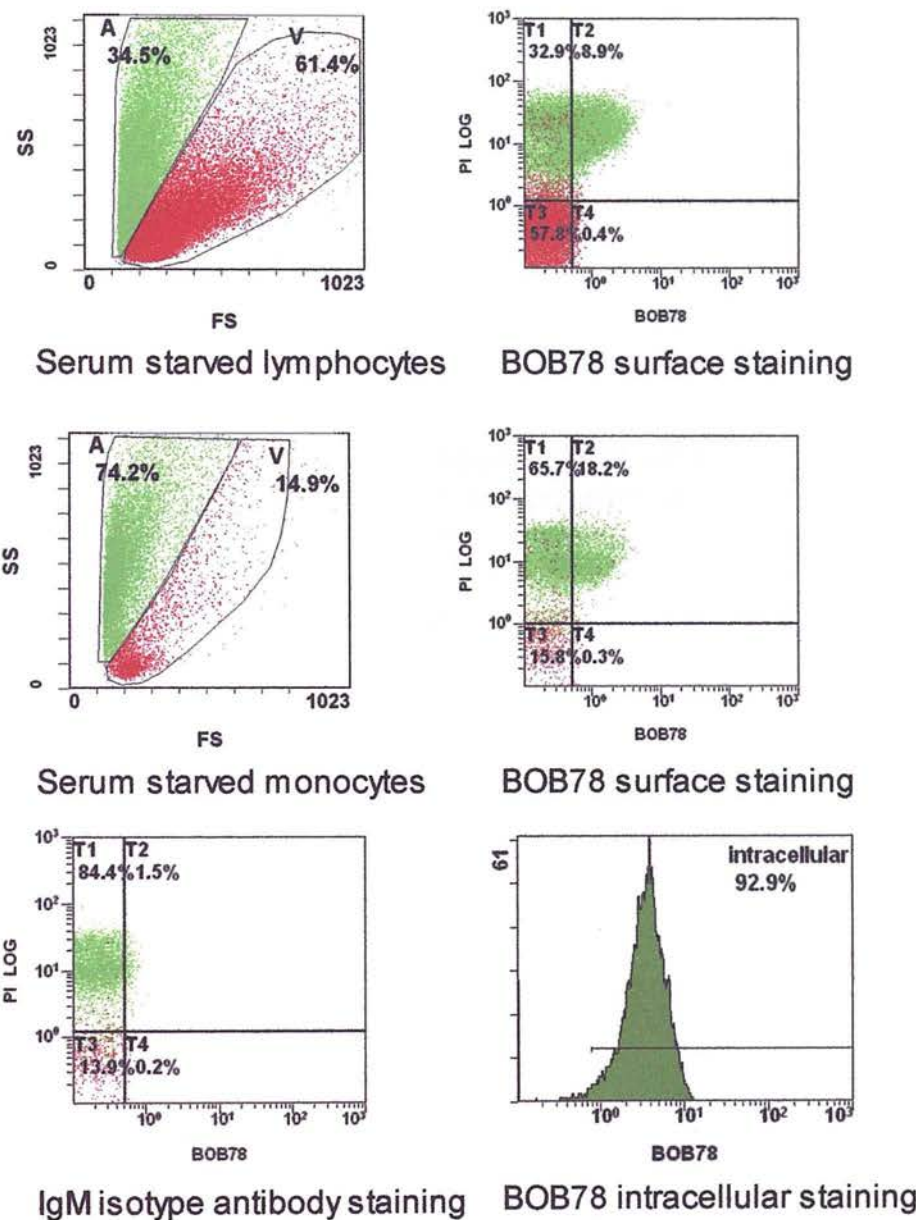
When peripheral blood monocytes and lymphocytes were studied for expression of BOB78, it was found that these non-cancer cells also expressed BOB78 antigen on the surface when undergoing apoptosis. In addition, on permeabilising the monocytes, intracellular expression of BOB78 antigen was verified (figure 4.1). This property is consistent with observations in leukaemic THP-1 and hepatoma HUH-7 cells (refer

Chapter 3). It appears that BOB78 antigen is not lineage-restricted within nucleated human cells. These results suggest that the BOB78 antigen may be highly conserved and a murine cell line was therefore tested for expression of BOB78 antigen. The mouse cell line SP2/0 was tested for the cytoplasmic presence of BOB78 antigen and the cells were confirmed as positive by flow cytometry (figure 4.2, A). It is interesting to note that dying SP2/0 cells expressed BOB78 antigen on the surface (figure 4.2, B). These results suggest that the antigen recognised by the BOB78 antibody is a highly conserved cytoplasmic antigen which is present in all nucleated cells. Since BOB78 is of IgM isotype, there is a possibility that the BOB78 antigen is a carbohydrate. Carbohydrates are recognised through pattern recognition receptors (PRR) expressed on macrophages as part of the innate immune system, and the process does not involve presentation of antigens to T lymphocytes. The helper T cells which direct heavy chain isotype switching to IgG class are therefore not involved (Abbas, Lichtman & Pober, 2000).

4.2 Searching for a tissue negative for BOB78 antigen

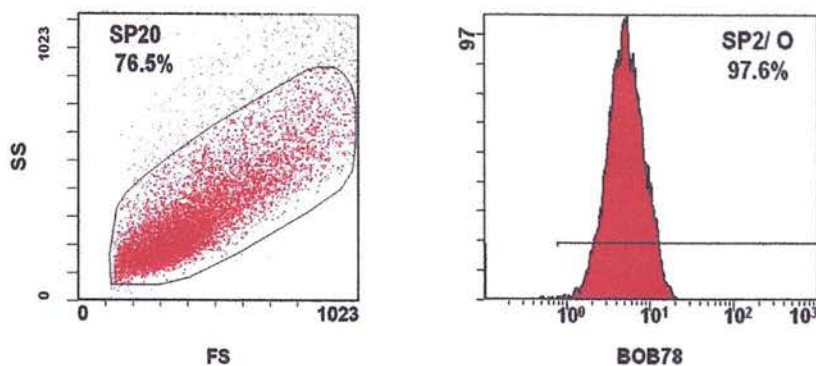
The ubiquitous distribution of BOB78 antigen within nucleated cells suggests that the molecule may serve certain highly conserved functions integral to the normal working of the cell. Moreover, the studies so far identify it as a surface feature of nucleated cells undergoing apoptosis. These observations suggest a possible dynamic role for BOB78 antigen at a time when cells are undergoing ordered deconstruction initiated by a highly conserved genetic programme. A cellular model where apoptosis as prescribed for nucleated cells does not apply might provide further evidence that BOB78 antigen is closely linked to the machinery of apoptosis. Human red blood cells provide such a tissue model. Mature erythrocytes are anucleate and are not equipped to undergo

Figure 4.1 Verifying presence of BOB78 antigen in normal leucocytes. Serum starved lymphocytes and monocytes were stained with BOB78 and IgM isotype control antibodies, with secondary anti-mouse FITC-conjugated antibodies. Analysis for surface expression was performed through flow cytometry. Cells were gated by size into dying cells (A) and viable cells (V).

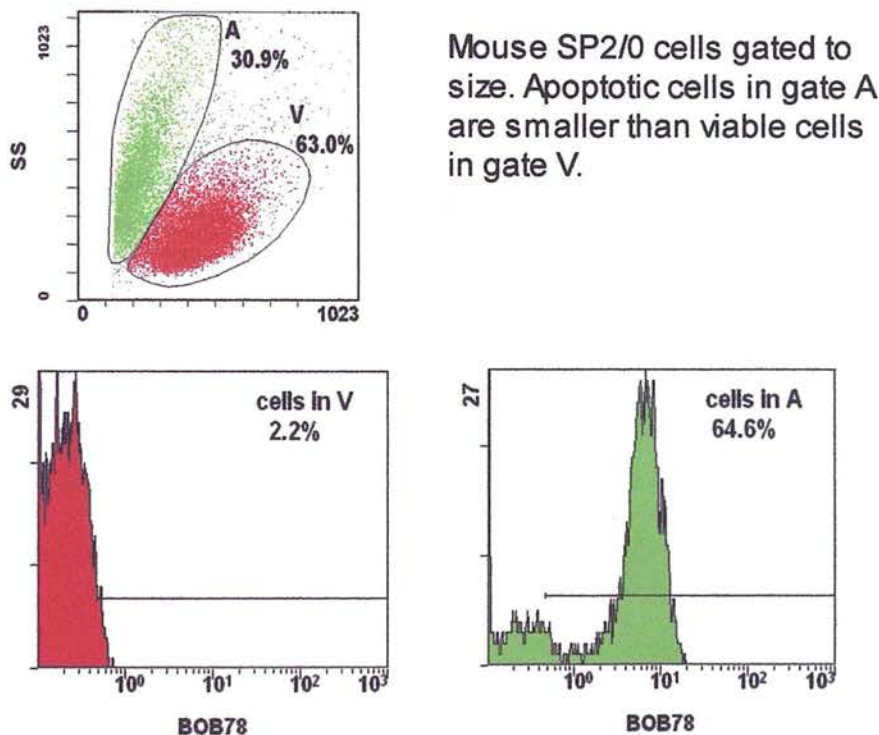


BOB78 antigen is expressed on dying leucocytes. Serum starved lymphocytes and monocytes expressed BOB78 antigen on the surface. BOB78 antigen is also present as an intracellular antigen in normal monocytes. Nucleated cells, tumour-derived or normal, appear to carry BOB78 antigen as part of normal cellular constituents.

Figure 4.2 Investigating the presence of BOB78 antigen in mouse SP2/0 cells. Mouse SP2/0 cells grown in standard medium were fixed and permeabilised, and stained for BOB78 (A). Another population of serum deprived SP20 cells were stained for surface BOB78 (B). Staining was detected with anti-mouse FITC-conjugated secondary antibodies and analysed through flow cytometry.



A: Intracellular BOB78 staining



B: Surface staining of BOB78 (FITC)

BOB78 is a multi-lineage antigen. Permeabilised mouse SP2/0 cells reveal intracellular BOB78 antigen. The ability of anti-human BOB78 antibody to detect mouse antigens agrees well with the fact that BOB78 is an IgM raised against a highly conserved antigen. The antibody consistently identifies apoptotic cells, in this case even mouse cells.

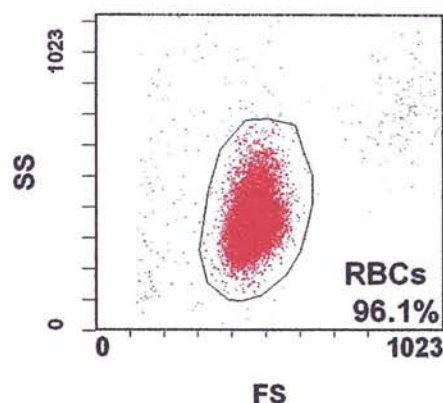
apoptosis *per se* like other nucleated cells (Weil et al., 1998). The presence or absence of the BOB78 antigen was therefore examined in erythrocytes. The red blood cells were fixed and permeabilised and stained for analysis by flow cytometry as described in Materials & Methods (chapter 2). Immunostaining was performed with anti-glycophorin as positive control, an IgM isotype negative control and BOB78.

4.2.1 BOB78 antigen is absent from erythrocytes – a cellular model negative for apoptosis

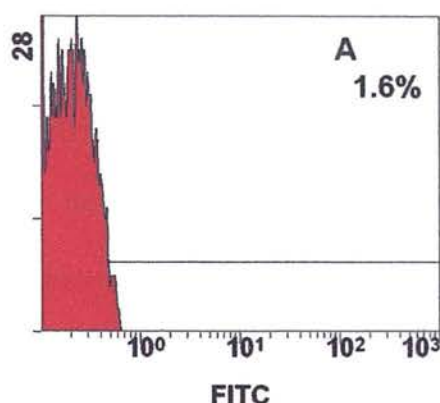
Normal erythrocytes expressed glycophorin on the cell surface but did not express BOB78 antigen. Upon permeabilisation, glycophorin within the erythrocytes was detected as before, there was however no staining for BOB78 (figure 4.3). The fixed and permeabilised cells had retained consistent morphology, and as the same permeabilisation techniques had been used to permit intracellular access of the IgM antibodies, the absence of BOB78 from erythrocytes was not likely to be artefactual. As the results obtained so far propose that the BOB78 antigen is highly conserved, the absence of the antigen from erythrocytes is of interest. Erythrocytes may thus qualify as a tissue negative for the BOB78 antigen although it is possible that a different isoform of the antigen exists, featuring a modified epitope not identified by the BOB78 antibody.

The findings so far propose that BOB78 is a highly conserved antigen which is ubiquitously expressed within the cytoplasm of nucleated cells. It becomes detectable at the surface of the plasma membrane when nucleated cells undergo apoptosis. It is possibly closely linked to the integral mechanisms of apoptosis.

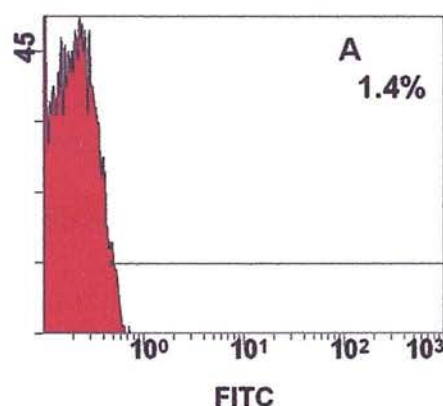
Figure 4.3 Erythrocytes – a tissue model negative for apoptosis. Erythrocytes were fixed, permeabilised, and stained with IgM control negative, BOB78, and glycophorin A (control positive) antibodies. Reaction of the primary antibodies was detected with secondary antimouse antibodies conjugated with FITC, and analysed on flow cytometry.



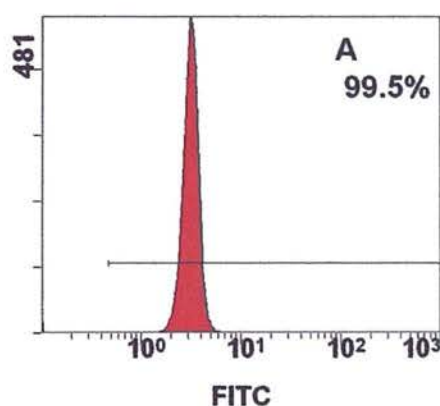
Erythrocytes maintained morphology after permeabilisation



IgM isotype control negative antibody



BOB78 antibody



Anti-glycophorin A, control positive antibody

The antigen BOB78 is not present in erythrocytes. The antigen BOB78 is absent from erythrocytes. As erythrocytes do not undergo apoptosis, this finding is consistent with the observation that BOB78 antigen is intimately linked to the orderly process of apoptosis.

4.3 Excluding carbohydrate as the epitope identified by the BOB78 antibody

The BOB78 antibody was confirmed to be of IgM isotype in earlier studies in our laboratory. This raises the possibility that the BOB78 antibody might be identifying a carbohydrate moiety such as sialic acid. Carbohydrates in the form of oligosaccharides are added to a host of proteins as a post-translational process and account for some of the activity and signalling capacity of these glycoproteins (Varki, 1993). Many of these glycoproteins reside on surface membranes or are secreted to effect these functions (Lippincott-Schwartz et al., 2000). Sialic acid is a very common oligosaccharide side chain in a host of mammalian glycoproteins (Karlsson, 1991). If a carbohydrate epitope is identified by BOB78, the antigen identified by BOB78 may be multi-specific, ie not specific for an individual protein species. This would seriously undermine the interpretation of the findings so far. It would also imply purification of a single BOB78 antigen with the antibody would be difficult. This issue was addressed with experiments using deglycosylating agents which remove oligosaccharide groups from glycoproteins. Removal of the oligosaccharide side chain would delete the positive detection of BOB78 if it is carbohydrate-specific. Positive antibody-antigen reaction however would not be affected if the BOB78 antibody is not identifying an oligosaccharide group. One of the most potent deglycosylating agents is neuraminidase-X from *Clostridium perfringens*. Reactivity of the BOB78 antibody before and after deglycosylation with neuraminidase-X was tested in the human leukaemic THP-1 cells, both in the intracellular site and when expressed on the surface. The dose of neuraminidase-X chosen was at a concentration whereby most major glycosylated groups especially sialic acid would be cleaved from glycoproteins.

4.3.1 BOB78 antigen within viable THP-1 cells remained detectable after deglycosylation

THP-1 cells were incubated in normal saline for one hour and treated with neuraminidase-X for 45 minutes or 90 minutes and the control population was incubated in normal saline for the same time period. The cells were then harvested and washed, and processed for flow cytometry after fixation in paraformaldehyde and permeabilisation in methanol as previously described (Chapter 2). The relative fluorescence from the different cells following flow cytometry was compared, as the level of fluorescence on flow cytometry is related to the quantity of antigen present (Schuerwegh et al., 2001). This revealed no appreciable loss in the positive detection of BOB78 after deglycosylating treatment with neuraminidase-X for 45 or 90 minutes (figure 4.4). The epitope recognised by the BOB78 antibody therefore does not comprise sialic acid. Moreover when THP-1 cells were first fixed and then permeabilised, the intracellular BOB78 remained detectable after treatment with neuraminidase-X which would have cleaved carbohydrate groups from glycoproteins (figure 4.5). To corroborate this finding further surface expression of BOB78 antigen on apoptotic THP-1 cells was also similarly tested before and after treatment with neuraminidase-X.

4.3.2. Surface expression of BOB78 antigen on apoptotic THP-1 cells remain detectable after deglycosylation with neuraminidase

Human leukaemic THP-1 cells were synchronised in S-phase of cell cycle with 2mM thymidine for 18 hours and induced to undergo apoptosis with serum deprivation for 2 hours (refer to Chapter 2). The cells were harvested, washed and incubated with neuraminidase-X in normal saline for one hour. The control population of apoptotic

Figure 4.4 Prevention of glycosylation in THP-1 cells with neuraminidase-X. Leukaemic THP-1 cells grown in standard medium were incubated in 154mM NaCl for 45 or 90 minutes, with or without treatment with neuraminidase-X. The cells were permeabilised and stained with IgM isotype control and BOB78 primary antibodies. Fluorescence on FITC-conjugated secondary antibody was analysed on flow cytometry.

BOB78 is not a carbohydrate moiety (sialic acid). There was no appreciable difference in the detection of intracellular BOB78 epitope after deglycosylating treatment with neuraminidase-X. The epitope on BOB78 is not a carbohydrate moiety.

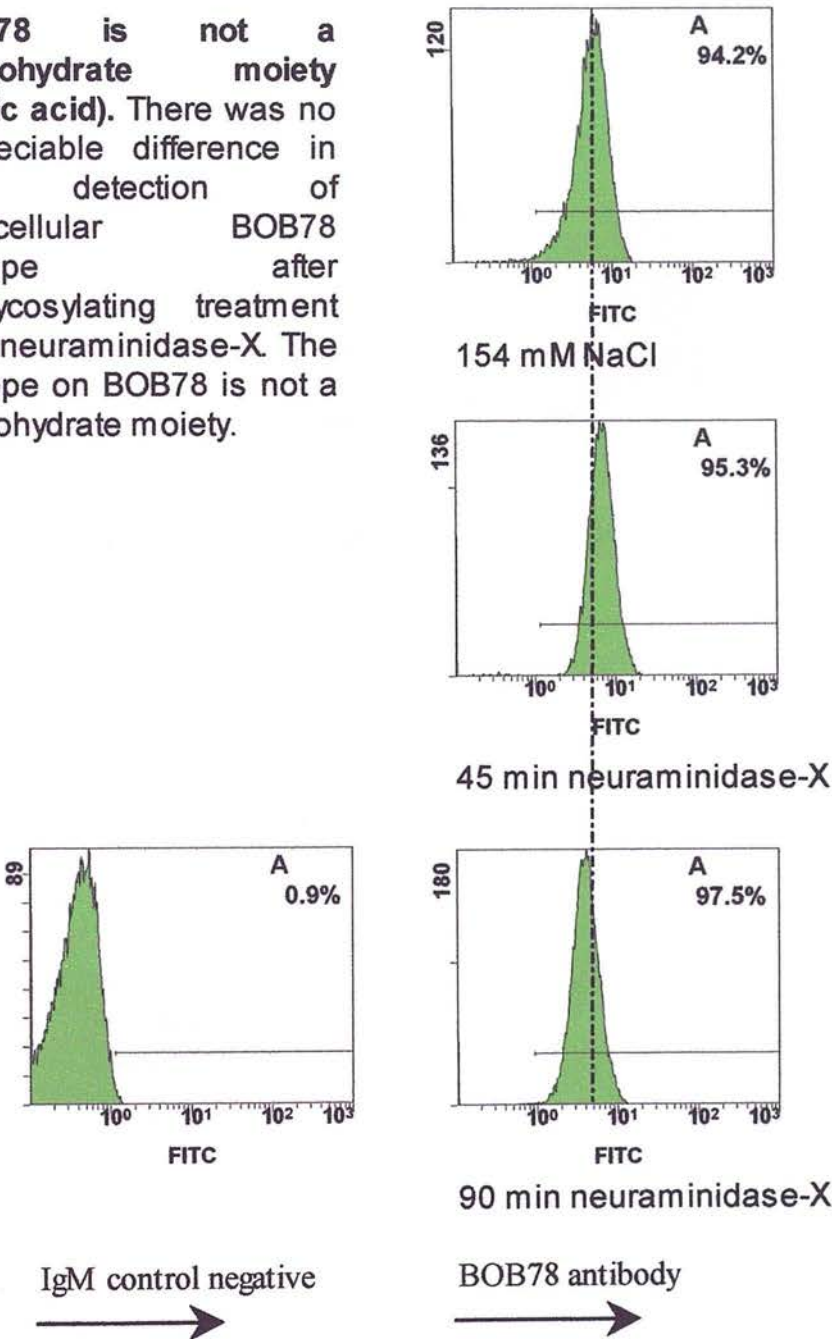
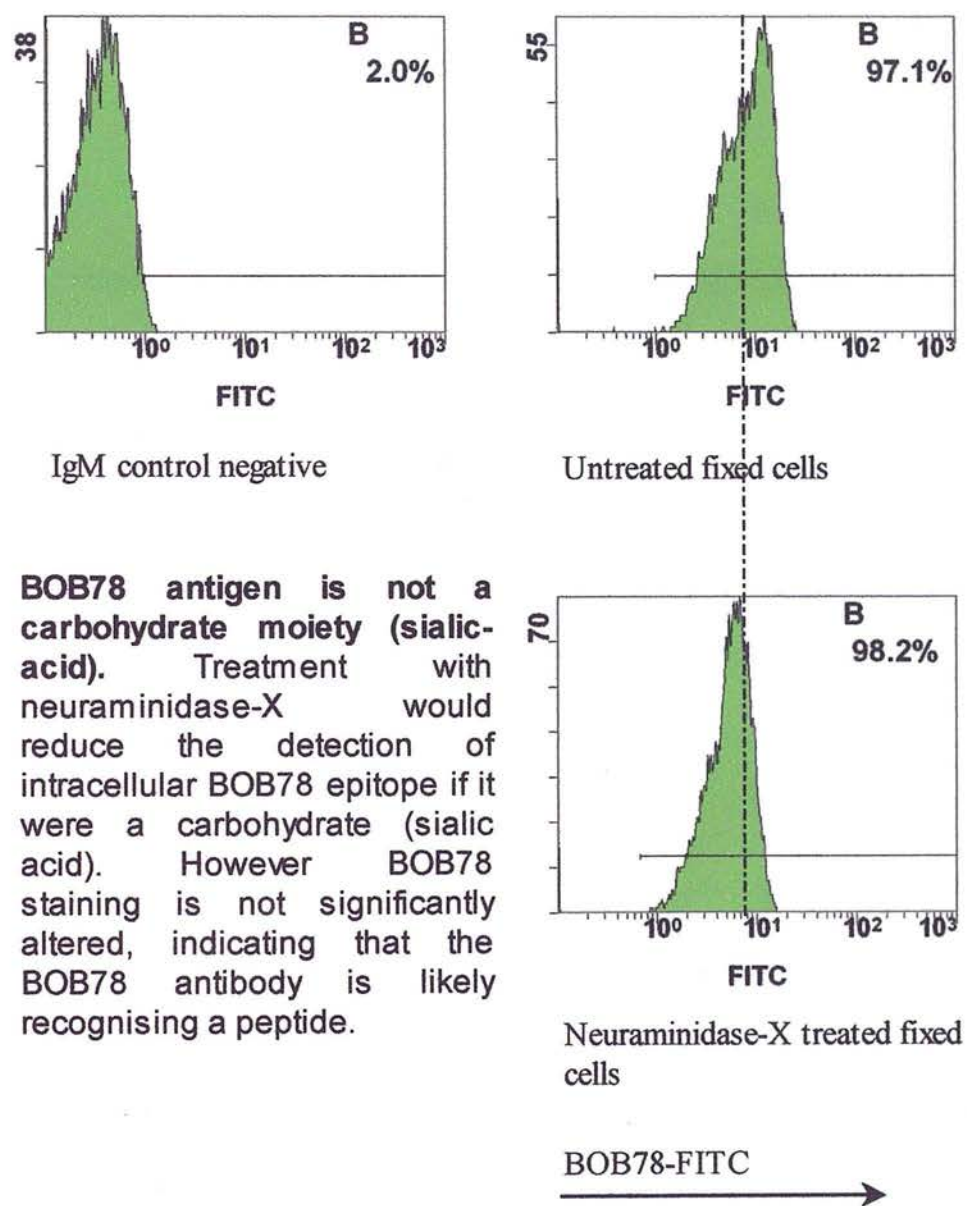


Figure 4.5 Deglycosylation of carbohydrate epitopes in THP-1 cells post-fixation. Fixed and permeabilised THP-1 cells were treated with neuraminidase-X for 90 minutes. The cells were then washed, stained with IgM isotype control and BOB78 primary antibodies. Fluorescence on FITC-conjugated secondary antibody was analysed on flow cytometry.



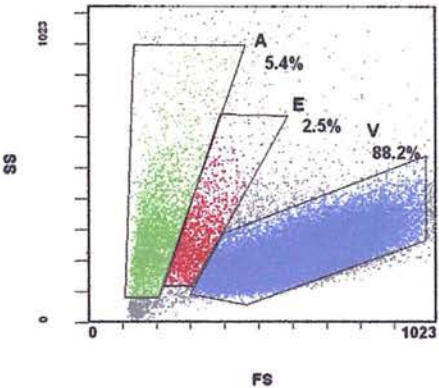
BOB78 antigen is not a carbohydrate moiety (sialic-acid). Treatment with neuraminidase-X would reduce the detection of intracellular BOB78 epitope if it were a carbohydrate (sialic acid). However BOB78 staining is not significantly altered, indicating that the BOB78 antibody is likely recognising a peptide.

cells was incubated with normal saline. The cells were then stained for BOB78 and analysed for surface expression of BOB78 with flow cytometry as previously described (Chapter 2). Analysis with the flow cytometer showed that BOB78 reactivity on the surface of apoptotic THP-1 cells did not change after treatment with neuraminidase-X (figure 4.6). The apoptotic cells were thus identifiable, as previously noted, as propidium iodide-dim but BOB78-bright cells. In addition, the proportion of apoptotic cells did not differ between the treated and untreated populations, indicating that treatment with neuraminidase-X did not compromise the viability of the THP-1 cells. These results agreed with the earlier observation that detection of intracellular BOB78 antigen was unchanged after deglycosylation. The BOB78 antibody is therefore not identifying sialic acid, a common carbohydrate moiety on mammalian glycoproteins.

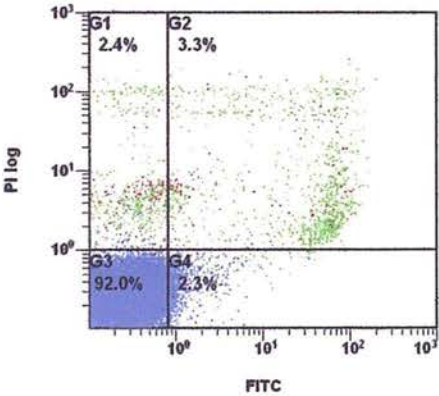
4.4 Excluding BOB78 antigen as a core oligosaccharide group

Having excluded that BOB78 is not identifying sialic acid, further glycosylation-related analysis was performed to evaluate if BOB78 is identifying an epitope related to the core oligosaccharide group in glycoproteins. Core glycosylation is the attachment of N-acetylglucosamine residues to the asparagine residue on the protein at the N-terminal. This is the first and obligate step in protein glycosylation (Abeijon and Hirschberg, 1992). Upon this carbohydrate structure are added other carbohydrate moieties specific to the glycoproteins. This core glycosylation step may be prevented by an antibiotic called tunicamycin (Chou et al., 1992). Tunicamycin is a hydrophobic analogue of UDP-N-acetylglucosamine. It blocks the addition of acetylglucosamine to dolichol phosphate, the first step in the formation of the core oligosaccharide. Incubating cells with tunicamycin would halt the maturation of glycoproteins, leading to breakdown in the normal biosynthetic-secretory pathway. If the epitope on BOB78 is related to core

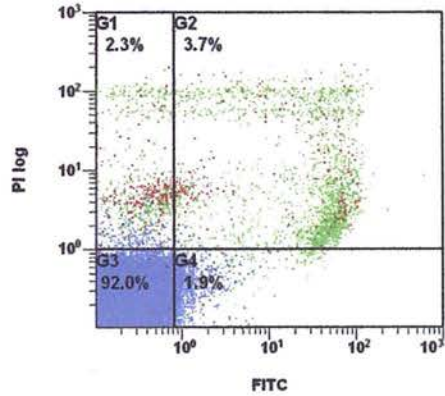
Figure 4.6 Detection of BOB78 on apoptotic cells after deglycosylation with neuraminidase-X. Human leukaemic THP-1 cells were induced to undergo apoptosis with serum deprivation for 2 hours. The cells were then treated with neuraminidase-X in normal saline, and processed for analysis of BOB78 surface expression on flow cytometry. Viable cells were separated from dying ones through propidium (PI) iodide staining



Separation of cells by morphology. Subpopulations of THP-1 cells were gated according to sizes. Dying cells were smaller and more granular than viable cells (V). There appeared to be a transition zone (E) bridging the main apoptotic subpopulation (A) and the viable group (V).



Cytometry with no neuraminidase-X treatment



Cytometry after neuraminidase-X treatment

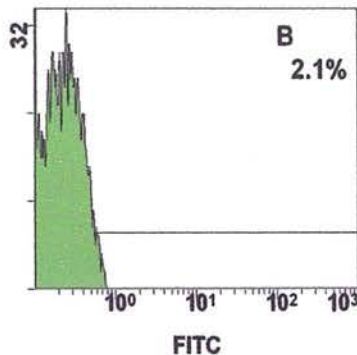
Identification of BOB78 is unaffected after deglycosylation. The panels above illustrate how the detection of surface BOB78 on apoptotic cells was unaffected after deglycosylation with neuraminidase-X treatment. This confirmed BOB78 is not identified through sialic acid residue, an oligosaccharide side chain prevalent among glycosylated mammalian proteins.

glycosylation, reactivity with the antibody would be reduced after incubation with tunicamycin. It is unlikely that detection of BOB78 antigen would be abrogated as the antigen appears to be present in abundant quantity inside the cells normally. However the shift in reactivity would be more pronounced if BOB78 antigen normally undergoes a high turnover rate. The effect of tunicamycin on intracellular BOB78 reactivity was investigated with human THP-1 cells using flow cytometry.

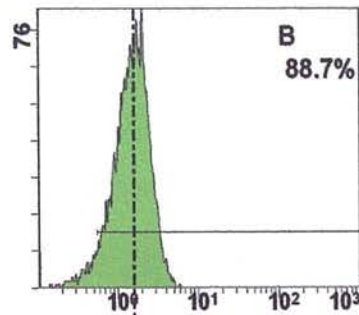
4.4.1 The epitope recognised by BOB78 is not related to a core oligosaccharide group

Human leukaemic THP-1 cells were cultured with tunicamycin over a dose range of 5 to 10µg/ml. The cells were then fixed and permeabilised, and processed for flow cytometry for detection of BOB78 as previously described (Chapter 2: Methods). Analysis with flow cytometry showed reactivity of the BOB78 antigen, reflected by the level of fluorescence, to be unchanged for cells cultured in tunicamycin when compared to untreated growing cells (figure 4.7). The peaks did not shift and had the same profiles as the untreated cells. This indicates that the BOB78 epitope is probably not related to the core oligosaccharide moiety. The incubation times would have affected biosynthesis of oligosaccharides on most glycoproteins, suggesting that BOB78 recognises a protein epitope. The antigen is not likely to undergo further processing within the ER-Golgi biosynthetic pathway. This possibility was examined further with Brefeldin-A, a Golgi disrupting agent.

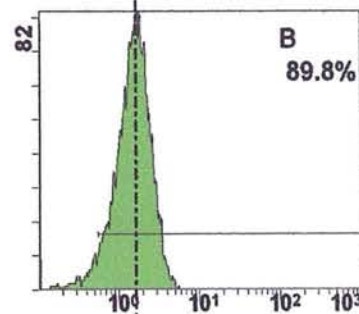
Figure 4.7 Inhibition of core glycosylation with tunicamycin. THP-1 cells were incubated with 5µg/ml or 10µg/ml tunicamycin for 18 hours to inhibit core glycosylation of proteins. The cells were washed, fixed and permeabilised. Primary antibodies were IgM isotype control and BOB78, with FITC-conjugated secondary antibodies. Fluorescence was analysed on flow cytometry.



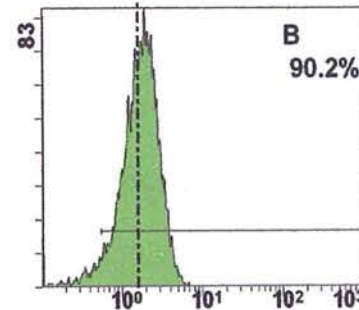
IgM isotype control



Untreated control cells



Tunicamycin 5µg/ml



Tunicamycin 10µg/ml

BOB78 epitope is not related to core oligosaccharides. Treatment with tunicamycin to inhibit core glycosylation did not reduce the detection of intracellular BOB78. This indicates the epitope detected by the BOB78 antibody is not a carbohydrate moiety.

BOB78 antibody →

4.5 Investigation of whether the BOB78 epitope matures within the Golgi apparatus

The results obtained from the experiments with neuraminidase-X and tunicamycin indicated that the epitope recognised by the BOB78 antibody is not sialic acid and probably not an oligosaccharide. Further characterisation of the BOB78 epitope was carried out with Brefeldin-A to investigate if the BOB78 epitope matures in the Golgi apparatus of the cell. Brefeldin-A is a fungal macrolide lactone. It is a small hydrophobic compound and a potent inhibitor of protein trafficking in mammalian cells (Sciaky et al., 1997). The effect on Brefeldin-A is interference with the formation of transport vesicles which traffic between the membrane systems of endoplasmic reticulum ER and the Golgi apparatus (Nebenfuhr et al., 2002). Brefeldin-A inhibits the formation of the coats that are required for the budding of the transport vesicles. Within these membranes the system transport vesicles bud from one compartment and fuse with the membranes of the target compartments thereby transferring proteins from one area to the next in the line of processing. This traffic is two-way with ER and Golgi apparatus freely exchanging vesicles and membranes. Within minutes of treatment with Brefeldin-A the Golgi apparatus disassembles and redistributes into ER. The endoplasmic reticulum may be seen to swell at higher doses of Brefeldin-A. This dramatic effect, which is especially evident on microscopy, occurs because, whilst Brefeldin-A specifically prevents the forward movement of transport vesicles from ER to Golgi apparatus, the normal backward movement from Golgi to ER is not affected by Brefeldin-A. This effect makes it a potent inhibitor of secretion of most glycoproteins which undergo terminal processing in the Golgi apparatus. When treated with Brefeldin-A, staining of BOB78 within normal cells would be affected if BOB78 antigen is an actively secreted entity or if the antigenic moiety on it depends on

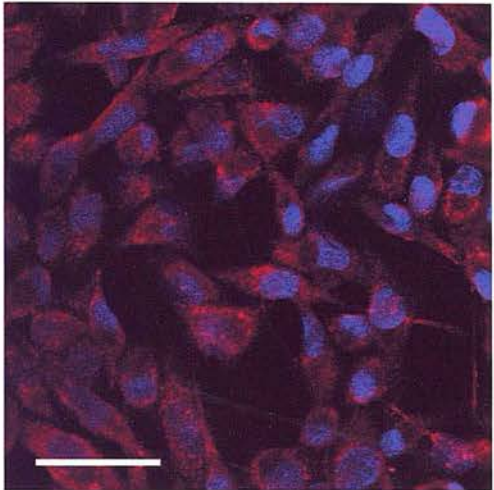
modifications within the Golgi complex. Intracellular stores of BOB78 antigen before and after treatment with Brefeldin-A were analysed with immunoblotting, whilst the effects on cytoplasmic turnover and distribution of BOB78 antigen were studied through fluorescence microscopy.

4.5.1. Brefeldin-A does not alter the epitope recognition of BOB78

The human pancreatic cancer Mia cells were grown in monolayers in chamber slides and treated with Brefeldin-A, with an untreated population acting as control (as described in Materials & Methods; Chapter 2). The cells were then washed, fixed and processed for immunocytochemistry staining for BOB78 antigen. When compared with the untreated population, treatment with Brefeldin-A did not render any clearly demonstrable effect on the cytoplasmic staining of BOB78. The cytosolic localisation of BOB78 antigen was unchanged (figure 4.8). The intensity of staining also appeared unchanged. Within the cytoplasm of the treated cells, there appeared to be no differential concentration of staining to any particular location which suggested compartmentalisation of the BOB78 antigen. The exact localisation of BOB78 antigen was difficult to determine within the distorted cytoplasm which was probably occupied mainly by the swollen endoplasmic reticulum membranes. The treatment with Brefeldin-A was sublethal as there was no apoptosis detected. These observations suggest that the BOB78 epitope is matured without the participation of the Golgi apparatus.

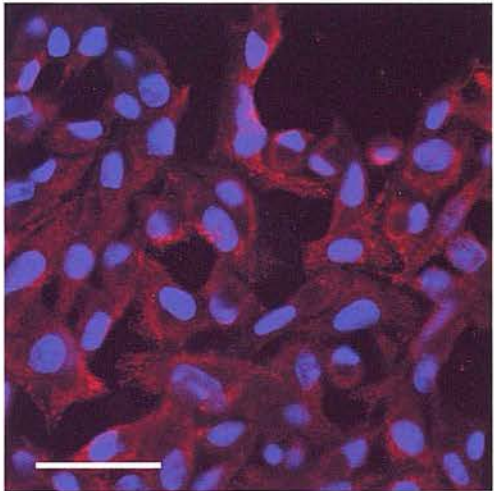
Figure 4.8 Effects of Brefeldin-A on BOB78 in Mia cells.

Human pancreatic cancer cells Mia were cultured in standard conditions and compared to incubations for 5 hours with Brefeldin-A at the annotated final concentrations. The cells were grown in monolayers in chamber slides, and were processed for immunocytochemistry with BOB78 and IgM isotype control primary antibodies. Secondary rabbit anti-mouse antibody was TRIT-C conjugated (red), with TOPRO-3 (blue) for nuclear counter-staining

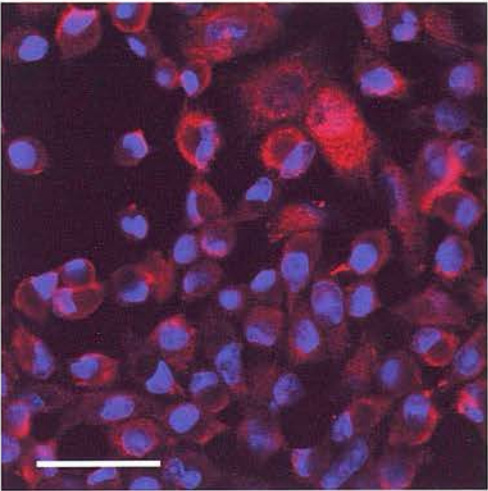


RPMI/10%FCS

BOB78 epitope is not matured in the Golgi apparatus. Staining for BOB78 persisted after treatment of Mia cells with Brefeldin-A which disassembles the Golgi apparatus. The pattern of cytoplasmic staining also appear unchanged. (Bar: 25µm)



Brefeldin-A 0.5µg/ml



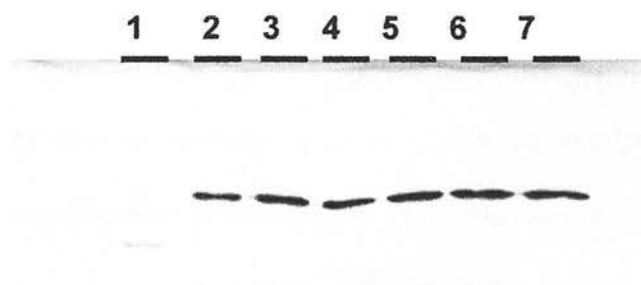
Brefeldin-A 5µg/ml

Another feature of the pattern of cytoplasmic staining of BOB78 also emerged from this study. Within both the untreated and treated populations of cells it appeared that BOB78 was not staining the Golgi apparatus. Whilst BOB78 was distributed throughout most of the cytoplasm there appeared to be a region relatively devoid of it (figure 4.8). This was the perinuclear zone of the Golgi apparatus (Hendricks et al., 1992). The cytoplasmic staining of BOB78 appeared to leave a perinuclear halo with no staining. In addition cells treated with higher concentrations of Brefeldin-A appeared to round up, which might be due to the effect of Brefeldin-A on cellular metabolism. The BOB78 antigen is therefore likely not a Golgi-related entity.

4.5.2. Brefeldin-A did not appear to affect the quantity of BOB78 antigen within the cells

An attempt was made to evaluate the possible effect of Brefeldin-A on the quantity of intracellular BOB78 antigen through the use of Western blotting. Human cells were treated as previously described (refer Chapter 2). The cells were then washed and lysed to yield protein lysate. The lysate was then subjected to Western blot as described in Materials & Methods (Chapter 2). The blots showed that the estimable quantity of BOB78 antigen as detected on Western blotting did not differ between the treated cells and the untreated ones (figure 4.9). The epitope detected by BOB78 antibody was probably not sensitive to Brefeldin-A treatment. This suggests maturation of the epitope does not occur within the Golgi apparatus.

Figure 4.9 Evaluating the expression of BOB78 after treatment with Brefeldin-A (a Golgi disrupting agent). Human pancreatic Mia cells were cultured in standard conditions, and compared with vehicle control (DMSO) and treatment with Brefeldin-A. The cells were lysed and BOB78 antigen was detected through immunoblotting with BOB78.



Brefeldin-A treatment did not affect expression of BOB78 antigen. The Western blot depicts the bands corresponding to BOB78 antigen. The density of the band is proportional to (but does not directly measure) the amount of antigen detected. There appears to be no dramatic change in the density of the bands, without or with treatment.

Bands labelled:

- | | |
|--------------------------------|-------------------------|
| 1: I -kB control positive | 2: untreated cells |
| 3: DMSO (vehicle control) | 4: untreated cells |
| 5: Brefeldin-A (BFA) 0.5 mg/ml | |
| 6: Brefeldin-A 5mg/ml | 7: Brefeldin-A 25 mg/ml |

It appears however that this technique may not be the most suitable for quantitative determination of the expression of BOB78 antigen within cells. The antigen is normally abundantly present within cells. Disruption to the functions of cells with Brefeldin-A treatment, sufficient in degree and extent to affect the quantity of such a ubiquitous protein, may very likely lead to serious functional stress and cell death. At the same time it had not been possible to synchronise the growth cycles of these cells. These confounding factors suggest that estimation of quantity of BOB78 antigen using immunoblotting may be prone to errors. This is probably true even if the turnover of BOB78 antigen is very high indeed or if it is somehow intimately linked to the membrane traffic of the biosynthetic pathways with its synthesis and breakdown closely related to those of other glycoproteins. This experiment with Western blot was therefore performed with no prior assumptions of the effects which Brefeldin-A induced.

4.6 Evaluating if movement of BOB78 antigen is affected by Monensin

The appearance of BOB78 antigen on the plasma membrane suggests that a targeting mechanism exists for this movement. It might be transported along the membranes involved in the biosynthetic-secretory pathway. In addition there is a possibility that BOB78 antigen might be secreted, if the antigen is intimately involved within this traffic system. The abundant expression of BOB78 antigen within the cells also prompts the question whether a proportion of the cytoplasmic pool might be involved in constitutive export to the plasma membrane. In order to study whether the BOB78 antigen might be exported by cells the fungal metabolite monensin was used. This antibiotic interferes with the export of glycoproteins. It has dramatic effect on the secretion of various cellular products including cytokines (Schuerwegh et al., 2001).

The effects of monensin on the turnover of glycoproteins may be studied through flow cytometry, where relative positive reactivities (mean fluorescence) may be compared (Schuerwegh et al., 2001). Should BOB78 normally be exported from the cell this disruption by monensin would manifest as increased signal on flow cytometry analysis (shift towards higher fluorescence). This glycoprotein export study was performed at the same time as the glycosylation studies with neuraminidase-X and tunicamycin.

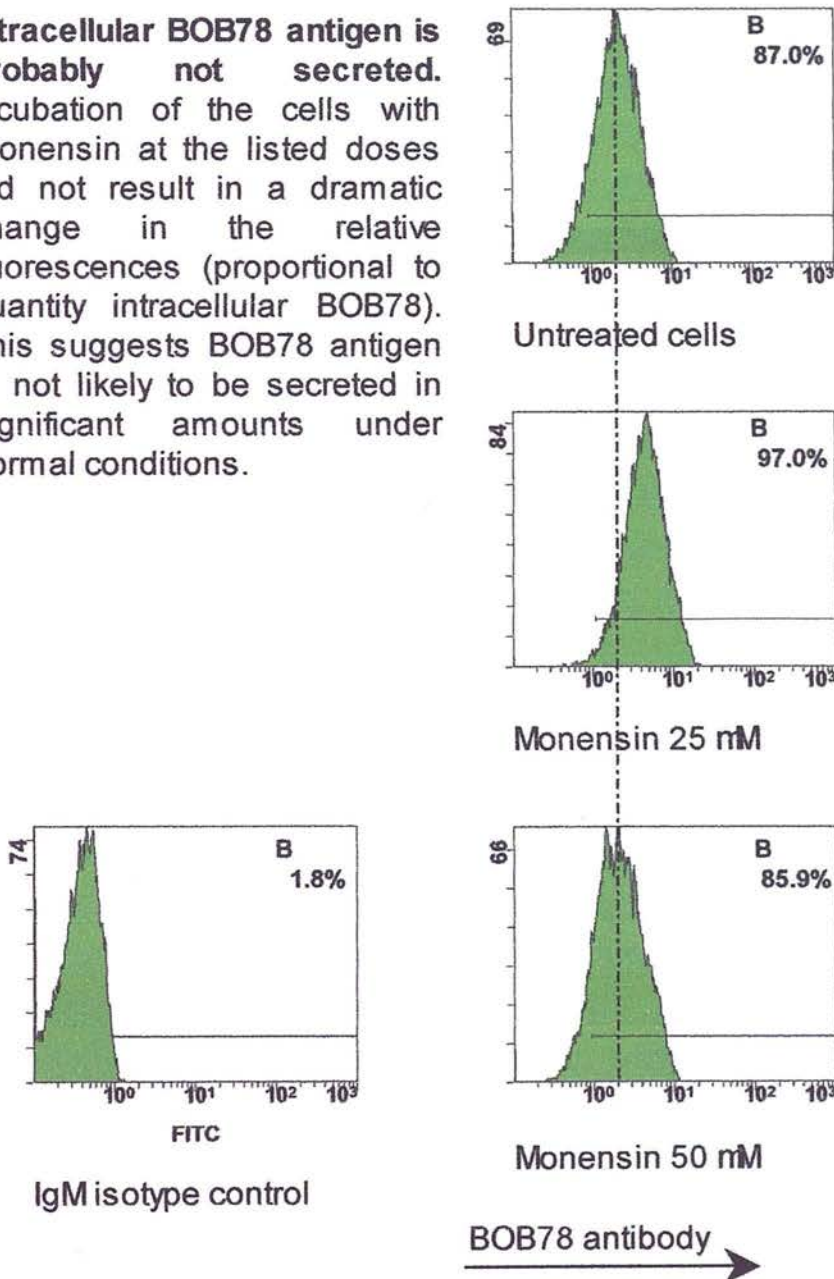
4.6.1. BOB78 antigen is probably not processed for secretion

The effects of monensin on intracellular BOB78 antigen was studied with flow cytometry. Other possible effects on the cytoplasmic pool of BOB78 antigen was followed with fluorescence microscopy. The human leukaemic THP-1 cells were grown in standard culture conditions and incubated with monensin at doses titrated to interrupt glycoprotein export without inducing significant apoptosis among the cells. Monensin was added during the log-phase growth of the cells, when turnover of cellular functions is high, for enhanced effects. The cells were then harvested and processed for flow cytometry staining for BOB78 as described (refer Chapter 2).

Positive detection of the BOB78 antigen remained unchanged after treatment with monensin. The relative fluorescences of both treated and untreated cells were similar (figure 4.10). The monensin treated cells did not exhibit a higher intensity of BOB78 fluorescence. This result concurs well with the finding that the epitope detected is not sialic acid, derived from the neuraminidase-X deglycosylation studies, as one of the mechanisms through which monensin interrupts glycoprotein export is by its inhibitory action on sialotransferases. The maturation of the BOB78 epitope is therefore not likely

Figure 4.10 Effect of monensin on BOB78 detection. Leukaemic THP-1 cells, cultured in RPMI/10%FCS at 1×10^6 cells/ml, were incubated with 25nM or 50nM monensin for 60 minutes or were untreated. The cells were fixed and permeabilised, and stained with primary IgM isotype control and BOB78 antibody, and FITC-conjugated secondary antibody. Fluorescence was analysed on flow cytometry. Control populations were compared with treated cells.

Intracellular BOB78 antigen is probably not secreted. Incubation of the cells with monensin at the listed doses did not result in a dramatic change in the relative fluorescences (proportional to quantity intracellular BOB78). This suggests BOB78 antigen is not likely to be secreted in significant amounts under normal conditions.



to be related to the activity of this enzyme and the BOB78 antigen does not appear to be secreted under normal circumstances.

4.6.2. Cytoplasmic distribution of BOB78 antigen appears unaffected by Monensin

The possible effects of monensin on cytoplasmic distribution of BOB78 antigen was examined by fluorescent microscopy. It was anticipated that use of cells with high basal secretory capacity and abundant cytoplasm might make any effects more apparent. The human pancreatic cancer Mia cells were selected for this experiment. The cells were cultured as monolayers in chamber slides and were incubated with monensin at doses ranging from 0.1 μ M to 100 μ M for 16 hours. Incubation with monensin was kept for a longer time period with the anticipation that dynamic changes within the Mia cells might be more evident. As Mia cells had been noted to be rather resilient to various forms of stress in culture, this amount of monensin was chosen even though doses within this range had been reported to induce apoptosis. The cells were then fixed and processed for immunocytochemistry as described previously (Chapter 2).

On immunocytochemical investigation the BOB78 antigen typically occupies most of the cytoplasm of normal cells. There is often a very thin rim of increased abundance of the BOB78 antigen at the nuclear envelope. There is no particular association with the edge of the cytoplasm or any cytoplasmic projections such as there is with some cytoskeletal proteins eg. cofilin (Bamburg and Bray, 1987). This pattern is reminiscent of the distribution of endoplasmic reticulum. The translocation of BOB78 antigen to the membrane blebs of apoptotic cells has been previously discussed in Chapter 3. There appears to be significantly less staining of BOB78 antigen within the nuclei of cells

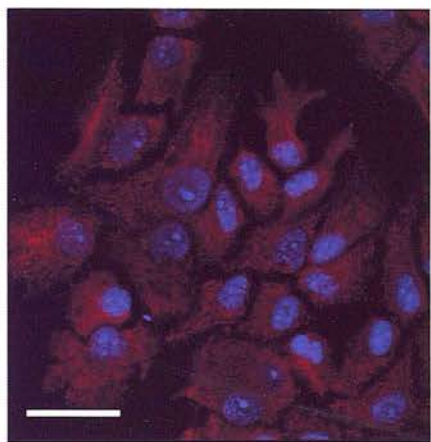
studied so far compared to the cytoplasm of live cells and the cell membranes of apoptotic cells (figures 3.13 - 3.17 and 4.8). On treatment with monensin as outlined above there appeared to be no dramatic effect on the cytoplasmic staining and distribution of BOB78 antigen (figure 4.11). The treated cells showed no dramatic change in the cytoplasmic localisation of BOB78 antigen even at high doses of monensin (figure 4.12). There appeared to be no particular concentration of BOB78 antigen to any area within the cytoplasm. The intensity of staining also appeared unaffected although this is not taken to infer an absence of change in the quantity of the BOB78 antigen. Apoptosis was not a significant feature of the treated cells.

An incidental finding with the inherently fast-growing Mia cells was that BOB78 was present in dividing cells (figure 4.11). The smaller cytoplasm of the dividing cells showed definite and rather intense staining for BOB78. It is not possible to comment whether the intense staining had any direct relation to the quantity of BOB78 as the cellular morphology and volume had changed.

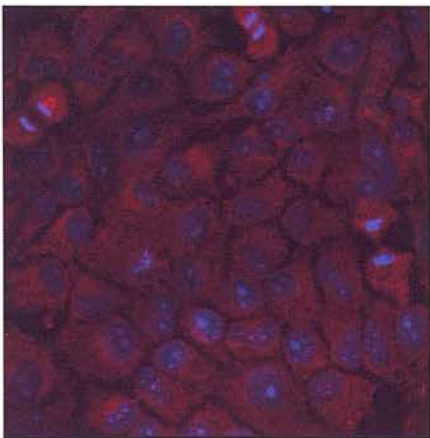
DISCUSSION

The results of the experiments detailed in this chapter allow a few conclusions about BOB78 to be made. It is evident that the epitope recognised by the BOB78 antibody is not sialic acid. The BOB78 antigen is also unlikely to be a oligosaccharide. There appears to be no demonstrable secretion of BOB78 from the cells under normal conditions. The antigenic site on BOB78 is very likely a peptide. Nevertheless it is still possible that the whole BOB78 molecule is a glycoprotein. Whilst tunicamycin may halt core glycosylation of the hypothetical "BOB78-glycoprotein" this does not change the antigenic recognition of the antigen by the BOB78 antibody. Tunicamycin would

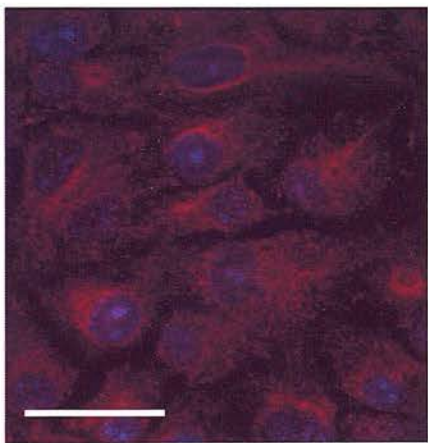
Figure 4.11 Effects of monensin on BOB78 antigen in Mia cells. Human pancreatic cancer Mia cells cultured in standard conditions and compared with 16-hour incubation with DMSO (volume adjusted vehicle control) and monensin at the final concentrations indicated in the legends. The cells were grown in monolayers in chamber slides and processed for immunocytochemistry with BOB78 and IgM isotype control primary antibodies. Secondary rabbit anti-mouse was TRITC-conjugated (red) with TOPRO-3 (blue) for nuclear counter-staining. (Bar: 25 μ m)



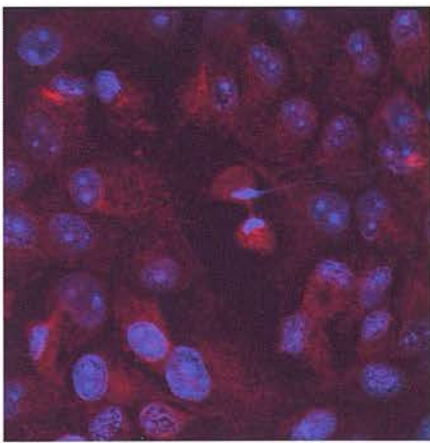
RPMI/10% FCS



DMSO (vehicle control)

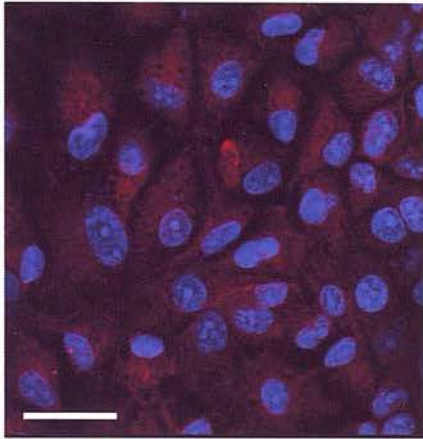


Monensin 0.1 μ M



Monensin 1 μ M

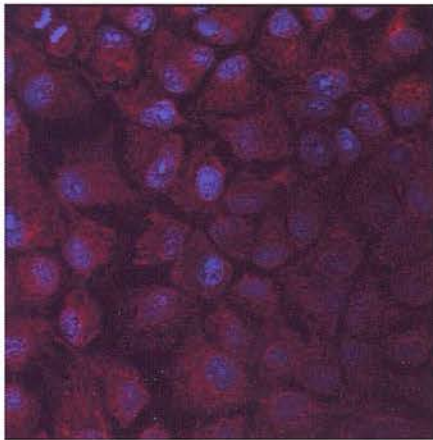
Figure 4.12 Effects of higher doses of monensin. Human pancreatic cancer cells were cultured with increasing concentrations of monensin as indicated in the legends and processed as described in figure 4.11.(Bar:25 μ m)



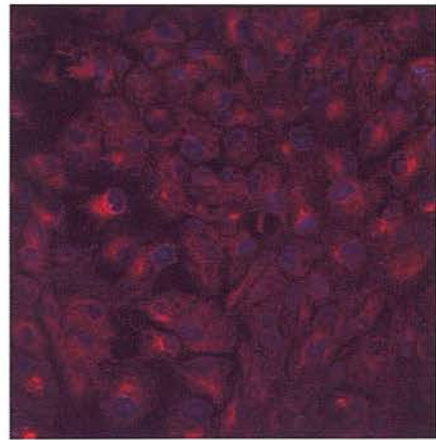
Monensin 5 μ M



Monensin 25 μ M



Monensin 50 μ M



Monensin 100 μ M

Titration of monensin concentrations does not affect cytoplasmic distribution of BOB78 antigen in Mia cells. Cytoplasmic distribution of BOB78 antigen appeared unchanged even at high levels of monensin.

however interrupt the onward traffic of this “BOB78-glycoprotein”. Since most glycoproteins are products of the biosynthetic-secretory pathway, interruption of this traffic would induce a change in the quantity and possibly cytoplasmic localisation of any glycoprotein. These effects are usually obvious on flow cytometry and microscopic analysis. If BOB78 is truly a part of a glycoprotein it is likely that its turnover within the cells is very low. This suggestion would not agree well with the likelihood of it being a glycoprotein.

Similarly treatment with monensin which inhibits glycoprotein movement and export should have a dramatic effect on BOB78 antigen if it truly is a processed for secretion. The abundant cytoplasmic pool of BOB78 antigen might account for the major proportion of the antigen detected, thus making any effects derived from incubation with monensin less apparent than anticipated. If this is the case, again as argued previously, the turnover of the hypothetical “BOB78-glycoprotein” had to be low. This appears rather incompatible with most secreted glycoproteins. Moreover the abundant cytoplasmic store of BOB78 antigen would probably be distributed into membrane-limited packages such as zymogens sensitive to signal-driven dynamics.

The results from Brefeldin-A treatment suggest that BOB78 antigen is probably not recruited into the Golgi apparatus under normal conditions in the cells. With its expression in the cytoplasm unchanged by Brefeldin-A it is very probable that the localisation of BOB78 antigen is closely related to the endoplasmic reticulum. Taken together with the studies with tunicamycin, monensin and neuraminidase-X, it can be tentatively concluded that BOB78 is a protein or a peptide. It undergoes very low

turnover and is not linked to the membrane transport of the biosynthetic-secretory pathway.

The pattern of cytoplasmic expression of BOB78 antigen on closer scrutiny with microscopy would propose that BOB78 may be an entity involved in the integral house-keeping functions in a cell. This proposal is borne out by the argument so far that it undergoes very little turnover and that it appears to be abundantly expressed at all times. Its presence within dividing cells would concur with this. This proposal would fit well with its likely intimate relation to endoplasmic reticulum ER. In normal cells ER partakes in a host of functions which range from biosynthesis of proteins and lipids, proper maturation and conformation of the proteins with its resident molecular chaperones, and synthesis of biomembranes which are destined for organelles and plasma membrane. This close link with ER would also be an avenue where BOB78 antigen may be transported to the plasma membrane.

The difficulty in synchronising the growth cycles of the cells has placed limitations on these kinetics studies and restrictions on the interpretations of the results. It had also not been possible to achieve near synchrony for apoptosis among the cells making it difficult to explore further queries of kinetics such as whether BOB78 antigen is actively synthesised or degraded during apoptosis.

The tentative conclusions are thus: BOB78 antigen is not an oligosaccharide, and probably undergoes low basal turnover. The next series of experiments which follow were attempted in order to isolate the BOB78 molecule for a more defined identity of BOB78 antigen and its functions. These are detailed in Chapter 5.

Although results from the experiments using neuraminidase-X, tunicamycin and Brefeldin-A consistently suggested that the epitope recognized by BOB78 is not a carbohydrate, validity of the observations would be consolidated if control positive experiments had been performed. Positive controls using glycoproteins could be used to verify the effectiveness of treatments with various agents used in deglycosylation and metabolic disruption of endosynthetic pathways. Certain glycoproteins which are known to be associated with the cell lines used eg. CD44 and CD15 would have been suitable candidates as positive controls, as they are likely to be affected by such treatments. Titrations of neuraminidase, tunicamycin, monensin and Brefeldin A would be guided by the loss of positive detection by appropriate antibodies directed against these glycoproteins in treated cells.

The possibility that the BOB78 antigen may be secreted was studied using monensin to block secretion of intracellular stores of glycoproteins. However, this could have been evaluated more directly by detection of the BOB78 antigen within the supernatant of cell cultures, using Western blots or ELISA.

CHAPTER 5

IDENTIFICATION OF BOB78 ANTIGEN AS A HEAT SHOCK PROTEIN

Part I. Investigating if BOB78 identifies Calreticulin

Part II. Purification and identification of the BOB78 antigen

INTRODUCTION

The results obtained so far indicate that BOB78 antigen is probably a protein, and likely a member of the house-keeping molecules integral to the normal functioning of a cell. The translocation of BOB78 antigen from the cytoplasmic pool to the plasma membrane may involve the endomembrane systems of endoplasmic reticulum, as plasma membrane is derived mainly from ER. Once positioned on the surface of an apoptotic cell BOB78 antigen remains stable until the cell undergoes late secondary lysis. The plasma membrane of a cell dying through apoptosis undergoes dynamic changes, amongst which include signals to mediate phagocytic recognition and engulfment. Externalised phosphatidylserine is an example of such changes. A possible role for BOB78 antigen which fits with the observations made in this project would be that of a molecular chaperone. Of the endoplasmic reticulum-associated chaperones, calreticulin (CRT) shares some striking similarities with the BOB78 antigen as elaborated below.

Calreticulin and BOB78 antigen: similarities

Calreticulin was first isolated almost 3 decades ago (Ostwald and MacLennan, 1974). CRT is found in all eukaryocytic cells, and is highly conserved with 90% amino acid identity among human, mouse, rat and rabbit (Michalak, 1996). It is a 46-kDa soluble protein, but has an apparent mobility on SDS-PAGE closer to 60-kDa weight mark (Michalak et al., 1992; Nash, Opas & Michalak, 1994; McCauliffe and Sontheimer, 1993). This is the calculated molecular weight of the BOB78 antigen on SDS-PAGE immunoblotting. Calreticulin is not found in erythrocytes (Krause & Michalak, 1997), a finding which matches that of BOB78. CRT has been shown to concentrate within the blebs of apoptotic cells. Keratinocytes induced to undergo apoptosis with ultra-violet irradiation generate discrete surface blebs which contain high concentrations of CRT (Casciola-Rosen, Anhalt & Rosen, 1994). CRT is an autoantigen prevalent in the autoimmune disease systemic lupus erythromatosus, SLE (Kishore et al., 1997). The CRT within the blebs possibly gets exposed to the adaptive immune system should the blebs be inadequately cleared following apoptosis. In fact, CRT had been found in these blebs together with other well- documented auto-antigens in SLE, the ribonucleoproteins (Staikou et al., 2003; Yang et al., 1999). Both BOB78 and CRT appear to share similar disposition within apoptotic cells.

In normal cells calreticulin is a molecular chaperone which localises mainly as a luminal protein in the endoplasmic reticulum (Opas et al., 1996). Molecular chaperones in cells prevent accumulation and aggregation of misfolded proteins which represent a threat to the homeostasis and viability of cells. In general molecular chaperones are proteins which binds to hydrophobic surfaces exposed in unfolded but not native

protein conformers, stabilising the folding intermediates, thereby preventing incorrect interactions (such as aggregation) and increasing the yield of properly folded protein (Bukau & Horwich, 1998; (Grallert and Buchner, 1999). This binding is reversible and involves ATP-binding and hydrolysis. Calreticulin had been confirmed to demonstrate chaperone activities for both glycosylated and non-glycosylated proteins (Saito et al., 1999). Observations from the experiments discussed in Chapter 4 have suggested that BOB78 may well be an endoplasmic reticulum -resident protein.

Calreticulin in its role as endoplasmic reticulum-resident chaperone is linked to the management of membrane-bound protein groups destined for the plasma membrane. CRT associates with the MHC Class I loading complex prior to peptide loading (Sadasivan et al., 1996). CRT had been shown to recognise misfolded MHC Class I proteins, and to possibly stabilise the loaded peptides within the MHC loading complex (Gao et al., 2002; Mancino et al., 2002) for presentation on the cell surface. CRT has recently been reported as being expressed on the cell surface of lymphocytes (Arosa et al., 1999). Apparently there is an increased amount of misfolded MHC Class I proteins in activated lymphocytes. It is these misfolded protein groups that CRT was associated with at the surface. The increased translocation of CRT to the surface was reported by Arosa and colleagues to parallel the increase of misfolded MHC Class I proteins. The ability of endoplasmic reticulum-resident chaperones to escape to the plasma membrane has been reported in several cell types (Wiest et al., 1997; Zhang et al., 1998). This traffic of calreticulin from its cytoplasmic (ER-membrane compartment) position to the plasma membrane of the cell is reminiscent of the translocation of BOB78 within a cell undergoing the dramatic changes of apoptosis.

RESULTS

Part I. Investigating if BOB78 identifies Calreticulin

5.1 Investigating if BOB78 antigen is Calreticulin

The above observations prompted studies to investigate if BOB78 might be identifying calreticulin or an epitope closely related in structure to calreticulin. Purified recombinant calreticulin was used to test if the BOB78 antibody might identify it. This was compared with the ability of an anti-calreticulin antibody to recognise the CRT antigen. The purified CRT protein was run on SDS-PAGE gel and blotted onto nitrocellulose membrane for probing with the calreticulin and BOB78 antibodies as described in methods (Chapter 2). Immunocytochemistry with MEG-01 cells was also performed to investigate the possible relationship between BOB78 antigen and calreticulin. The ability of calreticulin antibodies and BOB78 antibody to identify platelets generated from the human megakaryocytic cell line MEG-01 was also investigated. The use of this cell line is discussed in detail later in this chapter (refer 5.2). Briefly, it has been observed that platelet formation from MEG-01 cells involves the caspases which are employed in apoptosis (De Botton et al., 2002). The platelets are reminiscent of the blebs produced from the membranes of cells undergoing apoptosis (Stenberg and Levin, 1989). The results later in Chapter 5 detail the observation that the BOB78 antigen is being channelled into these platelets from the cytoplasm of the MEG-01 cells.

5.1.1. BOB78 does not identify Calreticulin

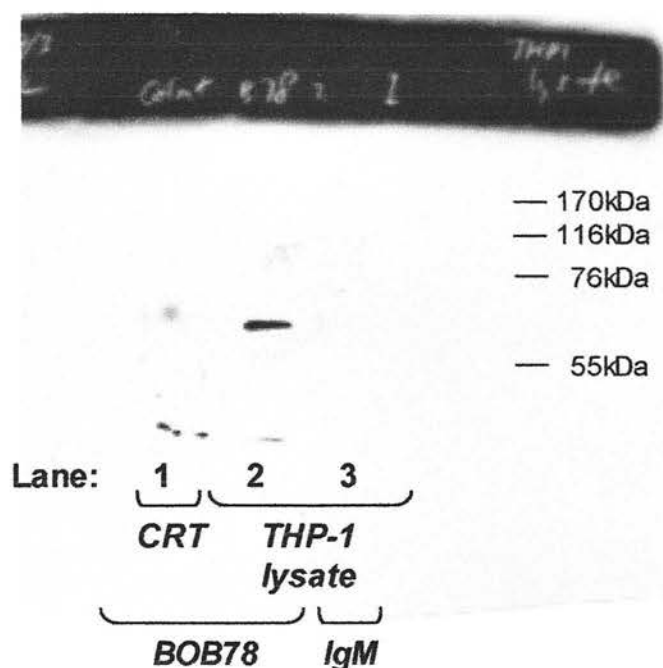
The purified recombinant human calreticulin protein was run on a SDS-PAGE gel, together with total protein lysate from leukaemic THP-1 cells and molecular weight markers. The separated proteins were then blotted onto a nitrocellulose membrane for probing by IgM isotype control and BOB78 primary antibodies. This reaction was detected through peroxidase-conjugated anti-mouse secondary antibody as described previously (Chapter 2). The results indicated that the BOB78 antibody did not identify the calreticulin antigen (figure 5.1). However it reacted with THP-1 cell lysate with a band corresponding to a molecular weight of approximately 65 kiloDaltons. This would mean that BOB78 is not an antibody identifying calreticulin.

5.1.2. The BOB78 antigen is not Calreticulin

Immunocytochemical analysis of the presence of BOB78 antigen and calreticulin within the human megakaryocytic MEG-01 cells was performed next. The MEG-01 cells were harvested and cytocentrifuged onto microscopic slides for immunocytochemistry as described (Chapter 2). Reactions of primary mouse anti-human BOB78 and IgM isotype control; and rabbit anti-human calreticulin and IgG isotype control, were detected with FITC-conjugated and TRITC-conjugated fluorescent secondary antibodies. The nuclei were counter-stained with TO-PRO 3. The fluorescent staining was analysed using both fluorescence and confocal microscopy.

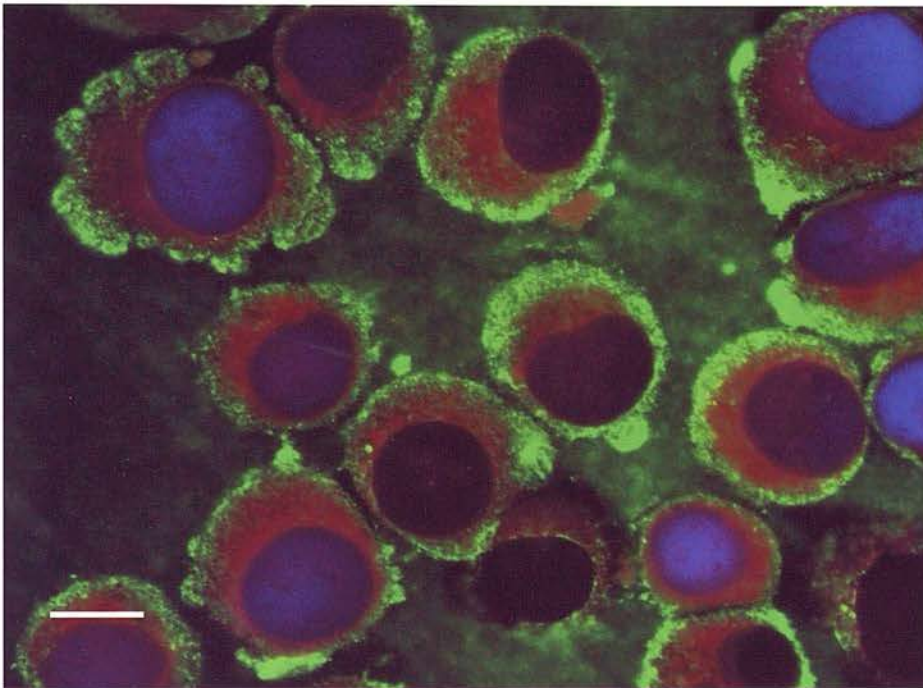
The results confirmed conclusively that BOB78 does not recognise calreticulin (figure 5.2). The most interesting feature was that BOB78 antigen was distributed in a location devoid of calreticulin. Their respective cytoplasmic distribution appeared mutually exclusive. The calreticulin present in MEG-01 cells was restricted to a band juxtaposed

Figure 5.1 Investigating if BOB78 identifies calreticulin (CRT). Total cell lysate from leukaemic THP-1 cells (lanes 2 and 3), and recombinant calreticulin (lane 1) were separated through SDS-PAGE and probed with BOB78 antibody. The immunoblot was developed with peroxidase-conjugated anti-mouse antibodies, and visualised through the ECL system.



BOB78 antigen is not calreticulin. The BOB78 antibody identified a 65 kDa antigen from the THP-1 cell lysate (lane 2). The purified calreticulin (lane 1) was not recognised by BOB78 antibody. An appropriate control for this experiment would be immunoblotting with anti-calreticulin antibody. This could confirm the presence of calreticulin and enable the molecular weight of calreticulin to be compared to that of the BOB78 antigen. The possibility of BOB78 identifying calreticulin was pursued further through immunocytochemistry with megakaryocytic MEG-01 cells.

Figure 5.2 Localising BOB78 and calreticulin in MEG-01 cells. Human megakaryocytic MEG-01 cells were cytocentrifuged into microscope slides and processed for immunocytochemistry. BOB78 (mouse) was detected with FITC-conjugated secondary (green), and anti-calreticulin (rabbit) was detected with TRITC-conjugated (red) secondary antibodies. Nuclear counterstaining was effected with TOPRO-3. The images were obtained as 3 separate frames under fluorescence microscopy, and merged with *Adobe photoshop*.



BOB78 does not identify calreticulin. BOB78 and calreticulin appear to be segregated to different intracellular locations. The BOB78 antigen is distributed to the outer cortex of the cytoplasm, whilst calreticulin is limited to the inner cytoplasm. This confirms the BOB78 monoclonal antibody is not recognising calreticulin. In addition BOB78 seems to be trafficked into the platelets budding off the cytoplasm. (Bar: 10mm)

to the nucleus. There appeared to be no extension of the rim of CRT to any other cytoplasmic locale. Just beyond this peri-nuclear band was the main distribution of BOB78 antigen. The outer cortex of cytoplasm was filled with BOB78. And there was no identifiable BOB78 within the rim of distribution of CRT. In fact BOB78 antigen was transported into the platelets of MEG-01 cells. The packaging of BOB78 antigen into the platelets appeared to be initiated as the cell membrane developed outward protrusions. The mutually exclusive distribution of BOB78 antigen and CRT within separate locale within the MEG-01 cells confirmed that BOB78 antigen is not CRT.

5.2 Studying BOB78 antigen in platelet production

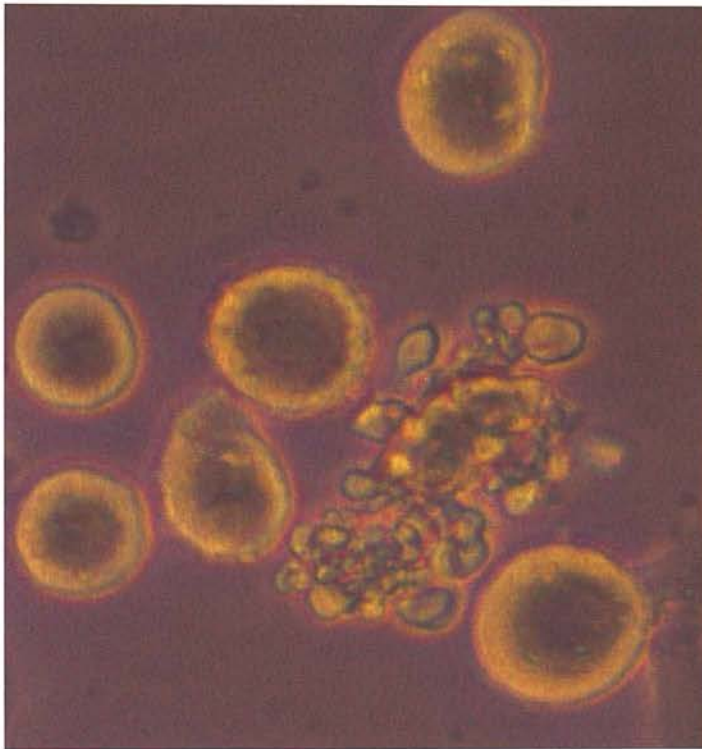
It has been demonstrated recently that caspases are involved in the generation of platelets from the human megakaryocytic MEG-01 cell line. Production of platelets arising from the terminal differentiation of MEG-01 cells was initiated by the activation of apoptosis-specific caspases (De Botton; 2002). Platelet production was inhibited by the (broad specificity) pan-caspase inhibitor z-VAD.fmk. However, once platelets began to form, such pan-caspase inhibition had no effect on the maturation and budding off of these platelets from the MEG-01 cells. This observation corroborates an earlier report that aged megakaryocytes undergo apoptosis and produce platelets (Zauli et al., 1997). The parent polyploid megakaryocyte cell body appears to involute through apoptosis and be engulfed by phagocytes in the bone marrow (Radley and Holder, 1983). Utilising human megakaryocytic MEG-01 cells which had been shown to produce functional platelets, Clarke and colleagues (2003) confirmed that these platelets were in fact generated through compartmentalised activation of caspases within the MEG-01 megakaryocytes. These platelets were devoid of caspase-9 although the lysate from these did contain caspase-3. These platelets undergo constitutive death,

displaying changes typical in apoptosis, leading to clearance through phagocytosis (Brown et al., 2000). The parent polyploid megakaryocytes proceeded to apoptosis after caspase-9 within them was activated. These various findings propose that the terminal differentiation of megakaryocytes leads to apoptosis of the parent cells, with activated caspases driving the dramatic cytoplasmic changes which culminate in platelets being budded off. Morphologically, a MEG-01 cell developing surface evaginations at the plasma membrane, which bud off into platelets, is reminiscent of an apoptotic cell developing membrane blebs (Takeuchi et al., 1998)(refer figure 5.3). This membrane blebbing in apoptosis has been shown to be an event which is dependent on, and consequential to, caspase activation (Coleman et al., 2001). Since the BOB78 protein has been demonstrated to be concentrated in the membrane blebs of apoptotic cells, it might also be found in the platelets derived from MEG-01 cells. This suspicion had been suggested by the immunocytochemistry studies localising BOB78 and calreticulin in MEG-01 cells (refer figure 5.2).

5.2.1. MEG-01 cells in the stages of terminal differentiation express BOB78 antigen on the surface

Human megakaryocytic MEG-01 cells in culture constitutively undergo terminal differentiation with concomitant production of functional platelets (Clarke et al., 2003). MEG-01 cells were maintained in standard culture conditions and were noted to have platelets which were shed into the culture medium whilst the parent cell went through morphological changes resembling those of apoptotic cells. The MEG-01 cells were harvested and processed for analysis by flow cytometry to evaluate surface expression of BOB78 antigen. The viability and stages of cell death of the MEG-01 cells was confirmed with propidium iodide staining.

Figure 5.3 Platelet formation in megakaryocytic MEG-01 cells in culture. Human megakaryocytic MEG-01 cells were maintained in standard cultures with RPMI/10% foetal calf serum. Photograph of cells in culture was taken through phase contrast microscope.



Membrane evaginations of MEG-01 cells reminiscent of blebbing in apoptotic cells. Megakaryocytic MEG-01 cells undergo terminal differentiation with production of platelets through protrusions in the cell membrane. These resemble the 'cytoplasmic boiling' observed in apoptotic cells where packages of cytoplasm would be delivered in membrane blebs.

The pattern of expression of BOB78 antigen on MEG-01 cells is highly reminiscent of the surface expression of BOB78 antigen on apoptotic cells (figure 5.4). The population of MEG-01 cells which are viable do not express BOB78 antigen on the surface and do not stain with propidium iodide. A proportion of MEG-01 cells which are smaller in size express BOB78 antigen on the surface. These are probably terminally differentiating MEG-01 cells which, having produced platelets, are left with scanty cytoplasm enclosing a polyploid nucleus. These cells exclude propidium iodide in the early stages of cell death when surface expression of BOB78 antigen is pronounced. In the later stages of cell death the surface expression of BOB78 antigen persisted whilst the cells became progressively more permeable to propidium iodide. However, cells decaying into secondary necrosis do not express BOB78 antigen on the surface membrane whilst being fully permeable to propidium iodide. This pattern of expression is akin to that demonstrated in apoptotic leukaemic THP-1 cells. It appears that BOB78 antigen is expressed by cells executing an inherent cell death programme.

5.2.2. BOB78 antigen is concentrated in the platelets of megakaryocytic cell line MEG-01

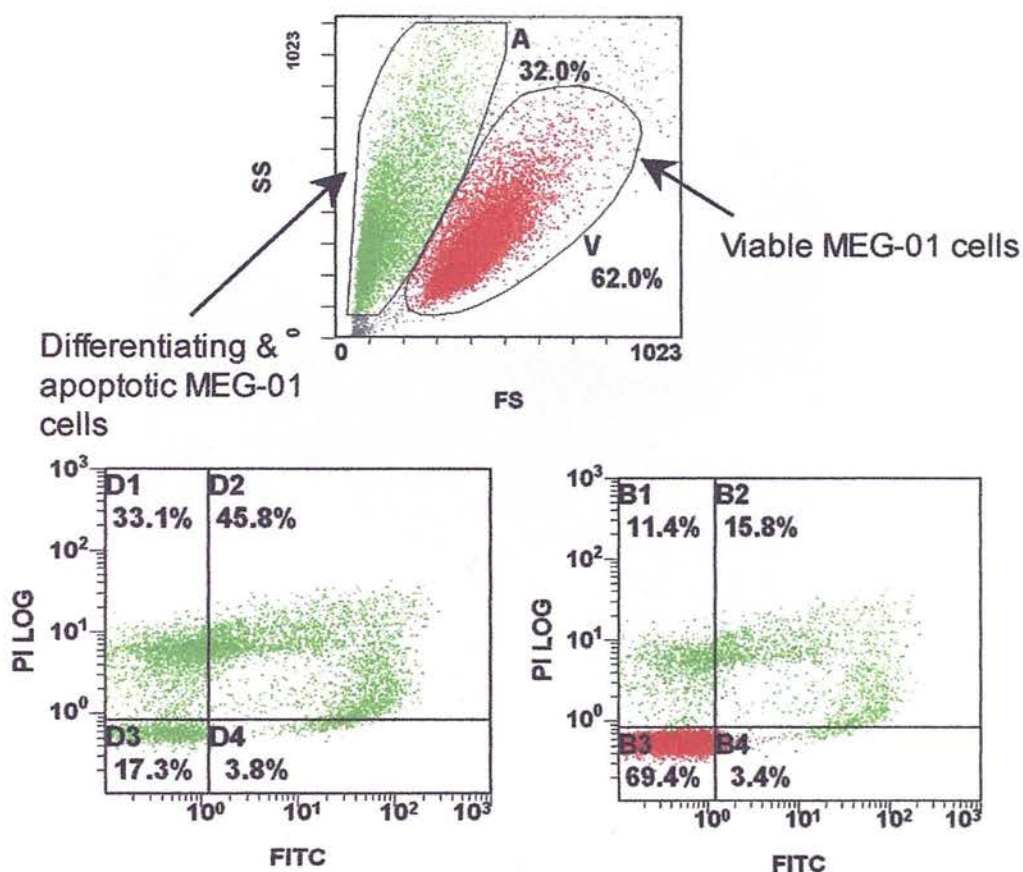
Megakaryocytic MEG-01 cells growing under normal conditions were harvested, cyto-centrifuged onto microscopic slides and processed for fluorescent immunocytochemistry as previously described (Chapter 2: Methods). The cells were stained for BOB78 antigen expression. Confocal microscopy revealed several striking features (figure 5.5). MEG-01 cells expressed BOB78 antigen within the cytoplasm. Closer scrutiny revealed the intracellular distribution of BOB78 antigen to be exclusively restricted to the outer cortex of the cytoplasm. The BOB78 protein from this outer rim of cytoplasm appeared to be trafficked into and sequestered within the

platelets as these pinched off from the parent MEG-01 cells. This phenomenon is highly reminiscent of the translocation of BOB78 antigen within blebbing apoptotic THP-1 cells (refer Chapter 3, figure 3.13). As discussed earlier, this movement of BOB78 antigen may be driven by the caspases which are activated during the terminal differentiation of MEG-01 cells. This observation corroborates the earlier observation that trafficking of BOB78 antigen within cells is linked to the initiation of the apoptosis programme. The MEG-01 cell bodies which remained after shedding of the platelets demonstrated absence of BOB78 antigen within the cytoplasm. The nuclei were also typically fragmented as in apoptosis (figure 5.5).

5.3 Expression of BOB78 antigen in senescent platelets

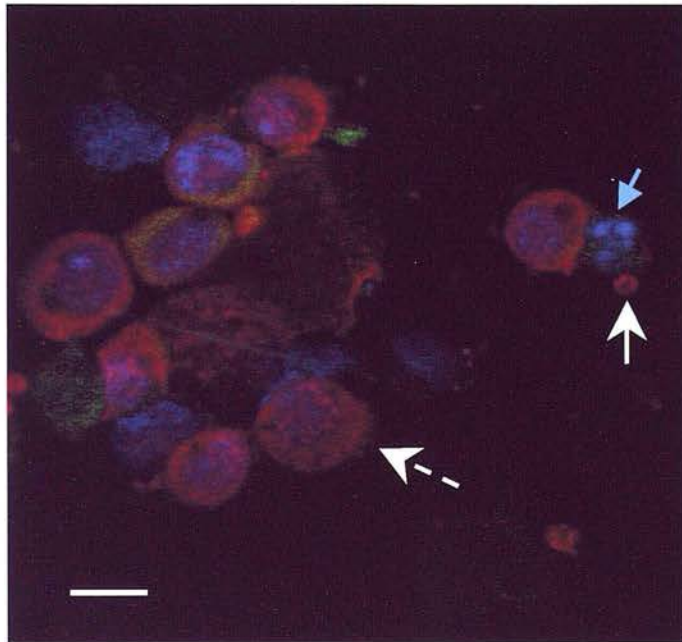
Platelets produced by the human megakaryocytic MEG-01 cells constitutively undergo senescence in culture (Brown et al., 2000). Effete platelets demonstrate externalisation of phosphatidylserine residues on the plasma membrane, just as apoptotic nucleated cells do. This membrane feature is a hallmark of senescence in platelets. These platelets contain caspase-3, caspase-9, Apaf-1 and cytochrome-c, at levels comparable to nucleated cells (Clarke et al., 2003). In addition the platelets also contain mitochondria. The MEG-01 platelets therefore carry an inherent programme of caspases and factors which may effect the intrinsic pathway of apoptosis. In other cells these caspases drive hallmark downstream events including flipping of phosphatidylserine to the outer leaf of the plasma membrane during apoptosis. Not surprisingly caspase-dependent DNase is absent from the platelets, as these are devoid of genomic nuclear material (Wolf et al., 1999). This key event of phosphatidylserine externalisation on the platelets was followed with Annexin-V binding and compared with BOB78 antigen expression on the surface to see if these are parallel events.

Figure 5.4 Expression of BOB78 in megakaryocytic MEG-01 cells. Human megakaryocytic MEG-01 cells were pooled from several days in standard culture conditions and processed for flow cytometry. Primary IgM isotype control and BOB78 antibodies were used, with FITC-conjugated secondary antibody. Viability of the cells was verified through exclusion of propidium iodide PI.



Apoptotic MEG-01 cells express BOB78 antigen on the surface. Terminal differentiation of MEG-01 cells culminates in apoptosis of the polyploidy parent cells, with the release of platelets. The remaining nuclei which had scant cytoplasm stained PI strongly in the late apoptotic stage. The differentiating MEG-01 cells which stain weakly for PI exhibit strong expression of BOB78 antigen on the surface, until the later stages of apoptosis. This profile is reminiscent of that with apoptotic THP-1 cells after treatment with camptothecin (CPT).

Figure 5.5 Localisation of BOB78 antigen to membrane blebs and platelets of MEG-01 cells. Human megakaryocytic MEG-01 cells were cytocentrifuged into microscopic slides and processed for immunocytochemistry. BOB78 (mouse) was detected with TRITC-conjugated secondary (red), and anti-calreticulin (rabbit) was detected with FITC-conjugated (green) secondary antibodies. Nuclear counterstaining effected with TOPRO-3. Fluorescence was analysed through confocal microscopy. (Bar: 15mm)

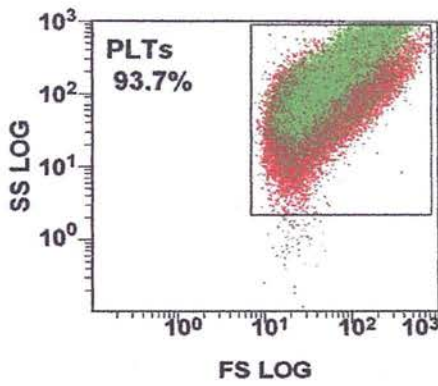


BOB78 antigen is concentrated in the MEG-01 membrane blebs and platelets. BOB78 antigen seems to be trafficked into the membrane blebs on the surface of MEG-01 cells (broken arrow). An apoptotic MEG-01 cell with chromatin clumps (blue arrow) appears to retain calreticulin in the cell body whilst the shed platelets (arrow) appear as subcellular particles with strong BOB78 staining.

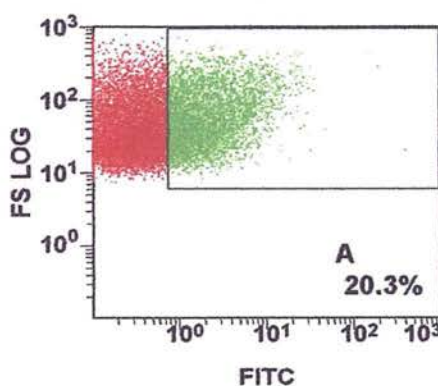
5.3.1 Senescent MEG-01 platelets express BOB78 antigen on the surface, as well as externalising phosphatidylserine residues

The platelets retrieved from the MEG-01 cell culture were processed and analysed for BOB78 antigen expression on flow cytometry as described in Chapter 2 (Methods). The platelets in culture were positively staining for Annexin-V in about 20% of the population. The aging subpopulation was distinguishable by generally reduced cell size on flow cytometry (figure 5.6). This same subpopulation also expressed BOB78 antigen on the surface (figure 5.7). The pattern of expression was not discrete, rather it appeared that the senescent platelets were at various stages of exposing BOB78 antigen over a continuum. The exposing of phosphatidylserine residues as detected by Annexin-V followed a similar pattern. It appears that the expression of BOB78 antigen on the membrane of these senescent platelets is concomitant with that of phosphatidylserine. This was similar to the coincident expression of BOB78 antigen and phosphatidylserine on the surface of apoptotic cells (refer to Chapter 3). The use of a vital dye such as propidium iodide to separate viable platelets from the senescent or dying ones was precluded by the absence of nuclei from platelets. The reported proportion of platelets in culture undergoing senescence is about 20 percent. However it was difficult to synchronise the maturation of MEG-01 cells, even in culture systems commencing with CD34 megakaryocytes in primary culture (Choi et al., 1995).

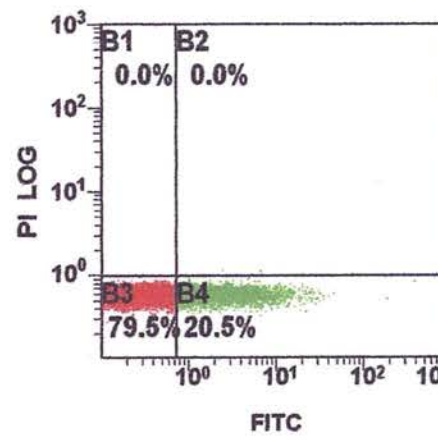
Figure 5.6 Analysis for senescence of MEG-01 platelets with Annexin V. Platelets produced by megakaryocytic MEG-01 cells were harvested from standard cultures and processed for flow cytometry. Senescence of platelets was assessed with FITC-conjugated Annexin V.



Senescent platelets (green) may not be distinguishable from normal platelets by changes in size on flow cytometry, although shrinkage of cytoplasm has been documented with electron microscopy.

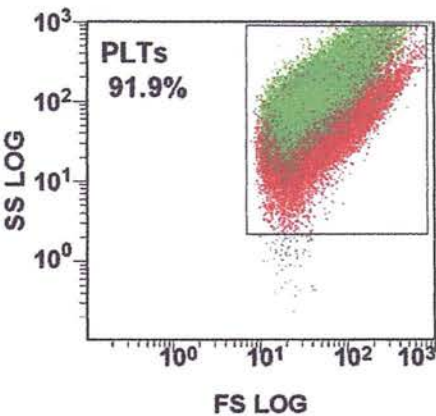


Aged platelets stain for Annexin V. Platelets from megakaryocytic MEG-01 cells undergo constitutive aging, with externalisation of phosphatidylserine on the plasma membrane. This is a feature of apoptotic nucleated cells.

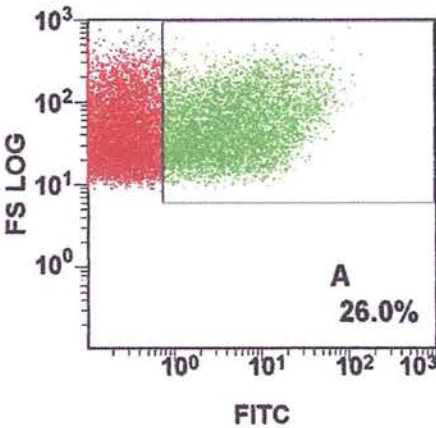


The standard dye exclusion test for viability with propidium iodide however would not differentiate the platelets as there is no nuclear material within them.

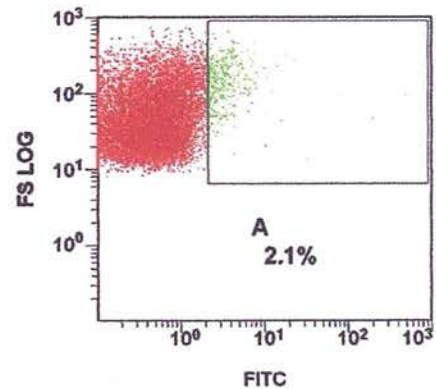
Figure 5.7 Expression of BOB78 in MEG-01 platelets. Platelets produced by megakaryocytic MEG-01 cells were harvested from standard cultures and processed for flow cytometry. The platelets were stained for surface expression of BOB78 antigen. Fluorescence from FITC-conjugated anti-mouse secondary antibody was analysed through flow cytometry.



The population of cells expressing BOB78 antigen on the surface appear to be similar to the senescent Annexin V-positive population (see figure. 5.6).



Senescent platelets express BOB78 antigen on the surface. This profile is similar to nucleated cells surfacing BOB78 antigen on the plasma membrane during apoptosis (see figure. 3.7).



Senescent platelets stained with IgM isotype control antibodies.

Part II. Purification and identification of the BOB78 antigen

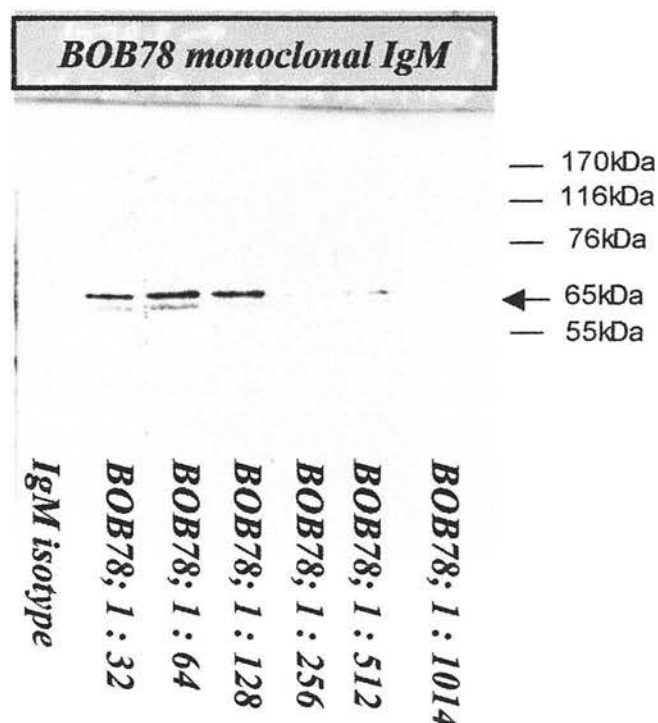
5.4 Determining the molecular weight of BOB78 antigen with SDS-PAGE and immunoblotting

The molecular weight of BOB78 was determined through mobility of the antigen in a SDS-PAGE gel compared with standard molecular weight markers. Lysate of human leukemic THP-1 cells was used in the SDS-PAGE as described in methods (Chapter 2). Briefly protein from a total cell lysate was loaded onto an acrylamide gel together with weighted molecular markers and driven through the gel by electrophoresis. The gel with the mass-separated proteins was blotted onto nitrocellulose membrane. This was incubated with control IgM isotype and BOB78 primary antibodies. Detection of the antigen by the BOB78 antibody was marked by a single specific band. The distance moved by the BOB78 band was compared with the distances moved by standardised molecular weight markers. The distances through which the molecular markers moved were plotted as an inverse linear relation ie. lighter proteins moved farther than the heavier proteins. The distance through which the BOB78 band moved was plotted off this curve to mark its weight.

5.4.1 BOB78 antigen is 65 kiloDaltons and this is consistent amongst various cell lines

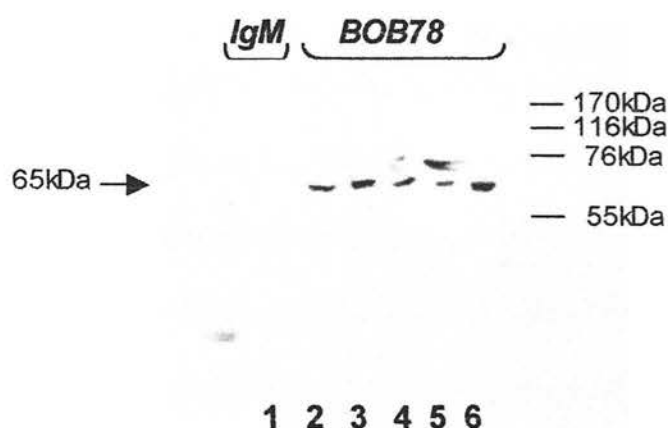
The molecular weight of the BOB78 antigen on SDS-PAGE gel was calculated as 65 kiloDaltons (figure 5.8). This weight was consistent when examined in other cell lines which included megakaryocytic MEG-01 cells, hepatoma HUH-7, HepG2 cells and pancreatic Mia cells. BOB78 antigen may be a highly conserved protein with a molecular of 65 kiloDaltons on a SDS-PAGE gel (figure 5.9).

Figure 5.8 The molecular weight of BOB78 antigen on SDS-PAGE. Lysate from leukaemic THP-1 cells was loaded in equal amounts (20ug per lane) onto a gel and the proteins were separated through SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane in semi-dry conditions. The BOB78 antigen amongst the proteins was probed with a titration of BOB78 monoclonal antibodies in decreasing concentration. Positive reaction was visualised through the ECL chemi-luminescence system.



The BOB78 protein weighs 65 kiloDaltons. From mobility on SDS-PAGE analysis with Western blotting the molecular weight of BOB78 antigen measured 65 kiloDaltons. This weight is calculated from a plot of the weights against the distances moved by the standard molecular weights. At titration of higher strengths, a band at a slightly lower molecular weight is apparent. This might represent non-specific reaction. Western blots were subsequently performed with BOB78 supernatant at 1: 100 dilution.

Figure 5.9 Investigating lineage specificity of BOB78 antigen. Total protein lysate from various cell lines were loaded in equal amounts and separated through SDS-PAGE and probed with BOB78 antibody. Western immunoblot was developed with peroxidase-conjugated anti-mouse antibodies, and visualised through ECL system. The numbered lanes were loaded respectively with proteins from MEG-01 platelets (lanes 1 and 2), MEG-01 cells (lane 3), THP-1 cells (lane 4), HUH-7 (lane 5), and Mia (lane 6).



Multi-lineage specificity of BOB78 antigen confirmed. The BOB78 antibody identified a 65kDa antigen from the various cell lines outlined above. Platelets generated from MEG-01 megakaryocytic cell line appear to have BOB78 in amounts not very different from the nucleated cells. This observation suggests the MEG-01 platelets might be a useful source of BOB78 antigen.

5.5 Isolation of BOB78 antigen through immunoprecipitation

Earlier attempts at isolating BOB78 antigen from neutrophils might have been impeded by breakdown of the protein by the proteolytic enzymes in these leucocytes (Hart et al., 2000). Having identified that platelets from the megakaryocytic MEG-01 cells were an enriched source of BOB78 antigen, these were used for isolating the BOB78 antigen through immunoprecipitation. To improve the capture of the BOB78 antibody which is a mouse IgM, an additional step using rabbit anti-mouse polyclonal antibodies to retrieve the BOB78 antibody-antigen complexes was employed. These secondary antibodies of IgG subclass were in turn bound onto Sepharose protein A, as described in Chapter 2 (Methods). The platelets were lysed to release the BOB78 antigen with a buffer containing Triton-X as membrane detergent. Triton-X had been noted to be less disruptive to the bond between the antibody and the antigen, which is non-covalent. The captured proteins as well as unreacted BOB78 antibody (hybridoma supernatant) were then separated on a SDS-PAGE gel under reducing conditions. One half of the gel was stained for proteins with Gelcode (colloidal Coomassie Blue, Pierce) whilst duplicate lanes on the other half were blotted and probed with BOB78 antibody in standard Western blot conditions. This was to confirm the specificity of the antigen capture from the platelet lysate. The weight of the band corresponding to BOB78 on Western blot was calculated from the standard molecular weights. The band on the stained gel which corresponded to this weight was then cut out for protein sequencing.

5.5.1. The antigen captured from MEG-01 platelet lysate is approximately 67 kiloDaltons

The reaction between the MEG-01 platelet lysate and BOB78 supernatant yielded a unique band at weight 67 kiloDaltons on staining with Gelcode. This band

corresponded to the specific capture of the BOB78 antigen (refer results 5.5.2). This was the band that was cut out for sequencing through MALDI-TOF.

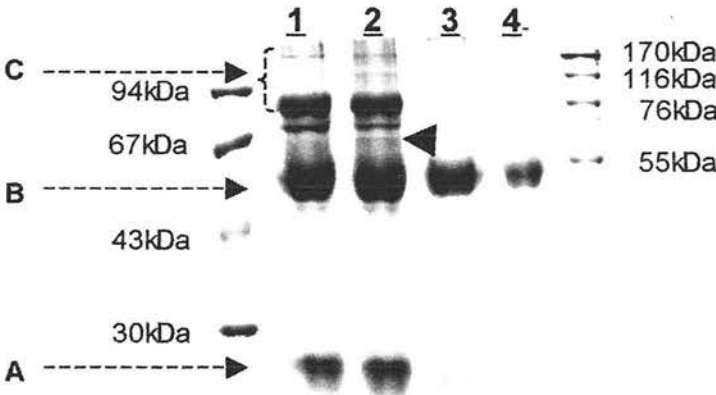
5.5.2. Antigen captured from MEG-01 platelet lysate is BOB78

The parallel half of the immunoprecipitation gel was probed with BOB78 antibody as per Western blot (described in Chapter; Methods). The residual non-specific activity of rabbit anti-mouse antibodies was quenched with an excess of mouse IgM isotype antibodies. The blot was then probed with BOB78 antibody. This showed a specific reaction at a weight of 67 kiloDaltons corresponding to the specific band antigen captured by the BOB78 supernatant from the MEG-01 platelet lysate.

5.6 Sequencing of the BOB78 antigen by MALDI-TOF

The immunoprecipitated proteins as well as the BOB78 antibody were separated through SDS-PAGE under reducing conditions. The SDS (sodium dodecyl sulphate) in the electrophoresis reagents would disrupt all non-covalent protein associations, thereby releasing the captured antigens from the antibodies. The separated proteins on the gel were stained with Gel-Code (Pierce), as this does not contain methanol which by reason of its chemical nature and reactions with the proteins and peptides, render mass spectrometry sequencing inaccurate. A band specific to the reaction between the platelet lysate and the BOB78 supernatant was identified and its weight was calculated at 67kDa (figure 5.10). The other half of the gel containing identical proteins had confirmed the specific capture of BOB78 and it had a weight of 67kDa on SDS-PAGE under these conditions (figure 5.11). The band was cut out and sent for sequencing using MALDI-TOF mass spectrometry. This sequencing service is run by Dr Andrew Cronshaw at the Edinburgh Protein Interaction Centre (EPICs). Protein sequencing was

Figure 5.10 Immunoprecipitation of BOB78 antigen. BOB78 antigen from the lysate of MEG-01 platelets was captured with the BOB78 IgM monoclonal antibodies. The immune complexes were then captured with rabbit anti-mouse antibodies, which in turn were captured by protein-A Sepharose beads. The captured proteins were released under reducing conditions and loaded into a gel for SDS-PAGE. The separated proteins were stained with Gel-Code (Pierce) and compared with the molecular standards in the same gel.



BOB78 antigen was precipitated from the MEG-01 platelets lysate. There was a band (arrowed) specific to the reaction between BOB78 monoclonal antibody and the platelets lysate, weighted at 67kDa. lane 2). This band was sent for sequencing with mass spectrometry (MALDI). Lanes 1, 3 & 4 represent control experiments.

Lane 1: BOB78 antibody supernatant; beads

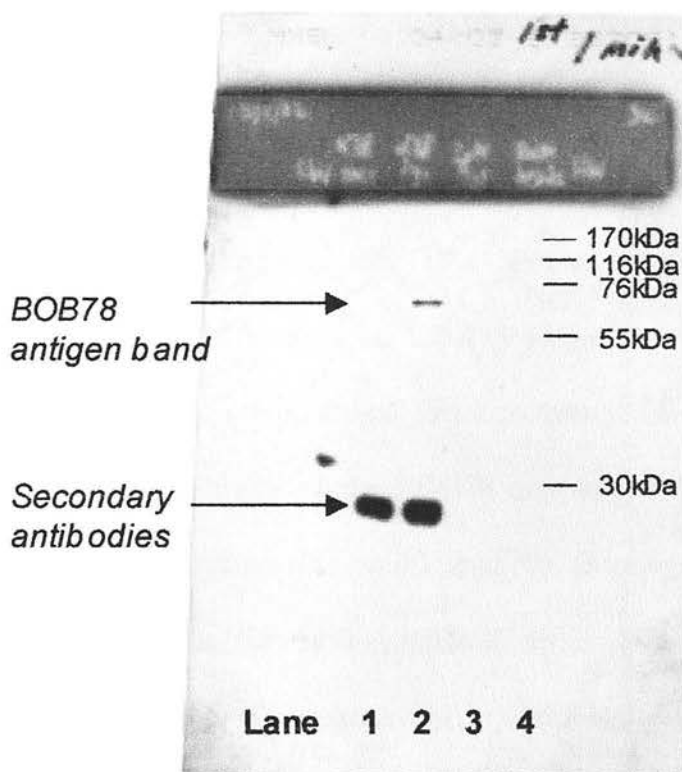
Lane 2: BOB78 antibody, platelet lysate; beads

Lane 3: IgM isotype control, platelet lysate; beads

Lane 4: platelet lysate; beads

Bands: A: immunoglobulin light chain
 B: immunoglobulin heavy chain
 C: other proteins in supernatant

Figure 5.11 Confirmation of capture of BOB78 at weight 67kDa. Duplicate lanes from the immunoprecipitate gel was probed with BOB78 antibody and processed for Western blot. The residual rabbit anti-mouse activity from the bridging antibodies was first quenched through incubation with an excess of blank mouse IgM molecules. The band indicated specific BOB78 antigen captured on immunoprecipitation. The band weighted about 67kDa.



The antigen BOB78 was captured at a band weighted at 67 kiloDaltons. The antigens captured through immunoprecipitation were identified with reactivity for BOB78 antibody. The lane of antigens captured from the reaction of BOB78 antibody with protein lysate yielded a band specific for BOB78 antibody, indicating specific capture of the antigen. A band of identical weight was identified on staining with Gel-code and was sent for MALDI-TOF mass spectrometry.

Lane 1: *beads incubated with only BOB78 antibody

Lane 2: lysate incubated with BOB78 antibody

Lane 3: lysate incubated with IgM isotype control antibody

Lane 4: lysate incubated with beads *(conjugated to rabbit anti-mouse antibodies)

performed on bands obtained from BOB78-platelet lysate immunoprecipitation on 3 separate occasions. These returned with the captured antigen positively identified as chaperonin, also known as heat shock protein 60 (hsp60) in mammalian cells (figure 5.12).

5.7 Verifying the MALDI-TOF sequencing with recombinant human heat shock protein 60, and commercially available mouse anti-human hsp60 antibody

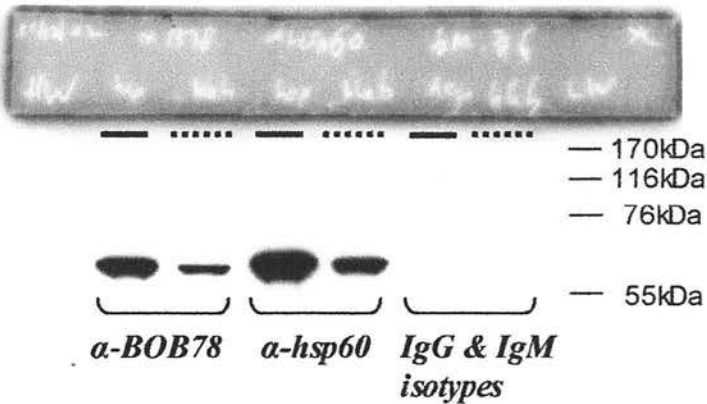
The putative identity of BOB78 antigen through MALDI-TOF sequencing was that of human heat shock protein 60, also known as chaperonin (figure 5.12). This identification of the antigen by BOB78 antibody was verified with commercially available recombinant human heat shock protein 60 (rh-hsp60, from StressGen). At the same time the reactivity of the BOB78 antibody was compared with commercially available mouse anti-human hsp60 antibody (Stressgen). Cell lysate from MEG-01 platelets and rh-hsp60 were separated on a SDS-PAGE gel and blotted onto nitrocellulose membrane as described in Chapter 2. The separated proteins were then probed with anti-hsp60 (StressGen) and the BOB78 antibody.

The Western blot showed that BOB78 identified rh-hsp60 at the molecular weight of 65 kiloDaltons on SD-PAGE (figure 5.13). This is also the weight of the BOB78 antigen consistently identified by the BOB78 antibody. Among the proteins from the MEG-01 platelets, the mouse anti-hsp60 antibody identified a band corresponding to a molecular weight of 65 kiloDaltons. This is the same weight for the band of purified recombinant human hsp60 identified by the anti-hsp60 antibody.

Figure 5.12 Summary of MALDI-TOF data

Sample Name	Identity	Mass (kDa)	% Coverage	MOWSE/ Peptides	Comments
9 Nov	BSA	69.3	28	1.6e+8 / 14	Good strong hit. Probable contamination from media.
15 Nov	HSP60 (Chaperonin)	61	23	4.0e+6 / 9	Good hit, again many peaks not matched. Over digestion / fragmentation?
19 Nov	HSP60 (Chaperonin)	61	29	2.3e+8 / 12	Strong but main peaks still not matched.

Figure 5.13 Verifying the reactivity of BOB78 against recombinant human (rh) heat shock protein 60. The reactivity of BOB78 for rh-hsp60 protein was compared with that of commercial anti-hsp60 monoclonal IgG. The lanes were loaded with either (recombinant human) rh-hsp60 (marked with thick line —) or lysate from MEG-01 platelets (marked with interrupted line). The proteins were separated on SDS-PAGE gel. Western blotting was performed with BOB78, anti-hsp60 monoclonal and isotype control antibodies.



BOB78 identifies heat shock protein 60. The BOB78 antibody identifies rh-hsp60 as well as BOB78 from MEG-01 platelets, at the same molecular weight. The purified anti-hsp60 monoclonal antibody identifies recombinant hsp60 as well as the BOB78 antigen, at the same weights as BOB78 detects these. No band of identification is detected with the IgM and IgG isotype controls.

DISCUSSION

Part I: BOB78 antigen and calreticulin

Calreticulin is a well-studied molecular chaperone important for many homeostatic functions in the cell (Michalak et al., 1999). It also sequesters calcium, a capacity vital to its homeostatic functions. There appear to be several similarities between calreticulin and BOB78 antigen, including absence from red blood cells. Studies using the human megakaryocytic MEG-01 cells have conclusively distinguished calreticulin from BOB78 antigen. BOB78 antigen (hsp60) is not calreticulin. The mutually exclusive distribution of these entities suggests different functions served by calreticulin and BOB78 antigen in MEG-01 megakaryocytes. It is interesting to note that calreticulin remains with the parent MEG-01 cell body after it has undergone terminal differentiation, releasing platelets in the process. *In vivo* these remnant apoptotic bodies of megakaryocytes are disposed of through phagocytosis by stromal cells in the bone marrow (Radley and Haller, 1983). This finding agrees with the implication of calreticulin having a role in phagocytosis of apoptotic cells (refer Chapter 1, Introduction). It would be reasonable to speculate a role for calreticulin in the clearance of these apoptotic bodies when the MEG-01 megakaryocytes have completed differentiation.

It is interesting how BOB78 antigen (hsp60) is exclusively concentrated within platelets whilst calreticulin is excluded from these. Both CRT and hsp60 are known to be cellular chaperones. Calreticulin has been implicated as a key moiety in recognition of apoptotic cells, being present on phagocytic cells (Ogden et al., 2001). Studies carried out in this research project have confirmed the expression of hsp60 on apoptotic

cells which may be important in the phagocytosis of apoptotic cells. It may be that, in megakaryocytes which are destined for apoptotic cell death, these chaperones with different functions are distributed according to the role they play with respect to the entities carrying them. Whilst calreticulin may be associated with the remnant cell body which is likely stationary within the bone marrow, the platelets which are due to be distributed within the circulation may be endowed with a predetermined life span. It is suggested that these two chaperones, calreticulin and hsp60, fulfill different functions within the same cell.

The detection of hsp60 on the surface of senescent platelets may have clinical implications. Currently platelets are kept at room temperature to prolong their shelf life. Interestingly it had been found that platelets are primed for activation when chilled (Hoffmeister et al., 2003). It had been noted that following activation, platelets undergo a clearance program driven by caspases, just like apoptosis. Quantifying the percentage of hsp60 positive platelets would allow an estimation of the amount of functional platelets. In addition such functional assays may allow various methods of storage to be investigated for optimal efficiency in prolonging shelf life. Such measure may also involve the use of caspase-inhibitors.

Part II: Purification of the BOB78 antigen

The megakaryocytic MEG-01 cell has been a fortuitous choice for characterising BOB78 antigen. Observations derived from the MEG-01 cells indicate that MEG-01 platelets could be a potent source of BOB78 antigen. This assumption is supported by findings with SDS-PAGE analysis of protein lysate from various cell lines. The use of MEG-01 platelet lysate has allowed capture of the antigen with the BOB78 IgM

antibody, a reaction which otherwise would have been rather more difficult. IgM immunoglobulins are noted for their low avidity for antigens (Medzhitov and Janeway, 2000).

From the findings derived from MEG-01 cells and platelets, caspases used by cells to drive apoptosis appear to be creatively mobilised to sculpture platelets out of terminally differentiated MEG-01 cells (De Botton et al., 2002). It is possible that through compartmentalised activation of the caspases, which act on the cytoskeleton, the body of a polyploid MEG-01 cell may be cleaved into discrete packages of cytoplasm as platelets. However, activated caspases are not packed into these platelets. Instead the complement of caspases able to drive the intrinsic apoptotic pathway are stored in these platelets until senescence occurs (Clarke et al., 2003). Senescent platelets undergo apoptosis with changes similar to the hallmark membrane changes of apoptotic cells. One of these changes is the expression of phosphatidylserine on the membrane. The phosphatidylserine residues are important for the pro-coagulant activities of platelets (Heemskerk et al., 2002). Senescent platelets also express BOB78 [i.e. hsp60] much like the parallel expression of BOB78 antigen and PS on the surface of apoptotic cells (see Chapter 3). These membrane changes may then translate into signals for recognition and engulfment by phagocytes. As senescent platelets expose BOB78 antigen on the plasma membrane, it may be inferred that the exposed hsp60 may form a component of the array of signals chemotactic to the phagocytes.

The antigen recognised by the BOB78 antibody had been conclusively been identified as heat shock protein 60, also known as chaperonin. The significance of this

observation and the possible functions hsp60 may serve on the surface of apoptotic cells are discussed in Chapter 6.

CONCLUSION

The antigen identified by the BOB78 antibody is conclusively identified as heat shock protein 60. It is a highly conserved protein in eukaryotes with homologues among the prokaryotes. We therefore conclude that BOB78 is chaperonin, heat shock protein 60.

CHAPTER 6

DISCUSSION

The aim of the project has been to characterise the antigen BOB78 as comprehensively as possible. The monoclonal IgM antibody BOB78 identifies an antigen expressed on the surface of apoptotic cells which has been positively identified as heat shock protein 60 (hsp60). The main conclusion derived from the studies arising from this project is that heat shock protein 60 is translocated to the surface of apoptotic cells. The significance of the findings and results detailed in previous chapters are discussed in three main contexts; 1) the nature of the antigen identified by BOB78; 2) the transport and the functions of BOB78 antigen on the membrane; and 3) the immunological significance of BOB78 antigen (hsp60) on apoptotic cells.

BOB78 identifies heat shock protein 60 on apoptotic cells

The antibody BOB78 was raised in our laboratory following immunization of mice with serum-deprived human monocytic leukaemic THP-1 cells. Hybridomas were created by fusing the mouse splenic cells with myeloma cells. Antibodies from the hybridoma clone coded as BOB78 were noted to bind to neutrophils in late apoptosis, as shown in earlier studies (Hart et al; 2000). Neutrophils aged in culture proceeded to apoptosis spontaneously. Whilst the surface expression of adhesion markers like CD16 was down-regulated, BOB78 appeared to be a prominent feature on the surface of apoptotic neutrophils. Interestingly, the BOB78 antigen was also demonstrated to be present in the cytoplasm of normal neutrophils. Isolation of BOB78 through immunoprecipitation however might have been rendered difficult by the inherent proteolytic

activities of the enzyme granules within the neutrophils, as Hart and his colleagues reported. The expression of BOB78 on the surface of neutrophils was suggested to be a late apoptotic event.

My studies confirmed the ability of BOB78 to identify apoptotic cells and further characterised its behaviour in these cells. The epitope recognised by the BOB78 antibody was successfully identified as belonging to heat shock protein 60.

Surface expression of BOB78 antigen (hsp60) is downstream to initiation of apoptosis

Analysis through flow cytometry marks the surface expression of BOB78 antigen as an event following the initiation of apoptosis. Using the lipophilic dye DiOC6, which is retained by functioning mitochondria, BOB78 has been shown to be expressed on the surface only after the mitochondria have depolarised (Ozgen et al., 2000). Functioning mitochondria maintain ionic polarity in the space between the inner and outer membrane, and the loss of this transmembrane potential is one of the key events in apoptosis. In the intrinsic pathway of cell death, this depolarisation initiates the activation of terminal caspases by releasing initiator caspases and other pro-apoptotic signals. Once the downstream effector caspases are activated cellular disassembly follows, with the substrates of the caspases including cytoskeleton proteins, DNAses, and nuclear laminins.

When a cell is in established apoptosis another key feature is the loss of plasma membrane symmetry. This results from the externalisation of phosphatidylserine residues which are usually retained on the inner leaflet of the membrane. These

exposed phosphatidylserine residues can be identified through their binding for another phospholipid called Annexin-V (Martin et al., 1999; Martinez and Freyssinet, 2001). The surface expression of BOB78 antigen coincides with that of phosphatidylserine. The expression of BOB78 antigen (hsp60) on the surface of the plasma membrane therefore occurs after apoptosis is initiated.

BOB78 antigen (hsp60) is expressed on membrane blebs of apoptotic cells

Using flow cytometry and fluorescence microscopy BOB78 antigen was shown to be expressed in the membrane blebs which develop on the surface of apoptotic cells. During apoptosis the plasma membrane develops evaginations called blebs to accommodate portions of cytoplasm which are extruded from the dying cell. Membrane blebbing with apoptotic bodies budding off a dying cell form some of the most distinctive features of programmed cell death (Kerr, Wyllie & Currie, 1974). Hence the earlier description of ‘cytoplasmic boiling’ of apoptotic cells, when these were studied with time-lapse photography. The membrane of an apoptotic cell maintains its semi-permeability, thereby sustaining the exclusive cellular environment where the cell death programme may proceed. Controlled deconstruction of the cell, with activities such as dramatic changes in cell shape, dissolution of the nuclear envelope, and degradation of DNA into small bundles, would not take place if the cellular integrity was disrupted.

Apoptotic bodies are small subcellular entities carrying various organelles and chromatin clumps, and are probably rendered into bodies of suitable dimensions in order to facilitate clearance. Whilst BOB78 antigen is usually expressed in the cytoplasm of the cell, during apoptosis the molecule appears to translocate to the

plasma membrane where blebs are developing. The membranes of developing apoptotic bodies as well as free floating ones are strongly positive for BOB78 antigen. The surface expression of BOB78 appears to be a distinctive and consistent feature of cells dying through apoptosis regardless of the methods of apoptosis induction. The observations imply that BOB78 identifies cells which are disintegrating through apoptosis.

Apoptotic cells are rapidly ingested by phagocytes *in vivo*. These may be neighbouring cells (non-professional phagocytes) or macrophages in the tissues (professional phagocytes). *In vitro* however apoptotic cells disintegrate or undergo lysis from secondary necrosis. This is expected from dying cells as most cellular mechanisms are shut down during apoptosis. Caspases which initiate and execute the cell death programme are not synthesised but are already present as precursors, ready to be activated, mobilised and recruited into action. Studies in this project with *in vitro* culture systems demonstrate that the surface expression of BOB78 antigen (hsp60) on dying cells persist up to the point where the cells begin to lose membrane integrity secondary to necrotic degeneration. When the membrane integrity is disrupted this surface feature is lost. This localisation of BOB78 antigen (hsp60) on the surface of cells throughout the apoptotic phase may have implications on the possible roles it may play in these cells and apoptotic bodies, for example, in recognition of apoptotic bodies.

BOB78 antigen (hsp60) is expressed on aged platelets

A creative deployment of caspases takes place in the generation of platelets from megakaryocytes (De Botton et al., 2001). In bone marrow precursors of

megakaryocytes fuse together to form multinucleated cells. Long and thin cytoplasmic extensions develop from the polyploid megakaryocyte during terminal differentiation. From these extensions cytoplasm is demarcated as packets which bud off as platelets. This process is apparently downstream to activation of caspases. The caspase-mediated disintegration of the megakaryocytic cytoplasm is followed by typical apoptotic involution of the nuclei; the platelets however do not show activation of caspases. Freshly produced platelets therefore do not exhibit membrane changes typical of apoptosis. Somehow the actions of the caspases are so compartmentalised that whilst the parent cell body dies, the progeny platelets remain as functional entities in that the hallmarks of apoptosis are absent. The platelets demonstrate the ability to undergo senescence during which they expose phosphatidylserine (PS) and are readily taken up by phagocytes (Brown et al., 2000). Phagocytosis is intimately related to PS on the plasma membrane. Platelets contain energy-producing mitochondria which may be induced to depolarise initiating classical downstream caspases activation and attendant PS externalisation. Albeit being anucleate, circulating platelets apparently contain the substrates to effect cytoplasmic and membrane changes akin to apoptotic termination when they are effete. Platelets are therefore capable of undergoing cell death through classical caspase-dependent pathways.

My studies with the megakaryocytic MEG-01 cells demonstrated that dying MEG-01 cells express hsp60 on the plasma membrane. These dying MEG-01 cells represent those which are differentiating to produce platelets. It is likely that where platelets are budding off the cell body hsp60 is expressed on the membrane. This may be because some of the platelets produced by MEG-01 cells are proceeding towards constitutive cell death prematurely. The remnant apoptotic nuclei at the final phase of terminal

differentiation of MEG-01 cells are devoid of hsp60 as studied on confocal microscopy. *In vivo* remnant megakaryocyte cells are cleared within the bone marrow stroma probably through phagocytosis by resident marrow stromal cells (Radley and Haller, 1983). In the bone marrow these apoptotic chromatin clumps may be disposed of differently from the way apoptotic bodies, containing organelles as well as nuclear material, are cleared from other tissues.

It is interesting to find that BOB78 antigen (hsp60) is also expressed by senescent platelets. This agrees with the observation of membrane expression of hsp60 being an event downstream to activation of initiator caspases. Since hsp60 appears to be exclusively packaged into platelets upon differentiation of megakaryocytic MEG-01 cells, it may be inferred that hsp60 forms part of the network of apoptotic machinery within the platelets. These hsp60 molecules are then mobilised to the plasma membrane when platelets undergo caspase-dependent cell death. The specificity demonstrated by BOB78 for hsp60 within the megakaryocytic MEG-01 platelets has made it possible to precipitate hsp60 from these.

Translocation of hsp60 onto the surface of the cell membrane

Heat shock proteins have been originally characterised for their up-regulated expression in bacteria exposed to heat shock. They are synthesised under other conditions of cellular stress, so are also known as stress proteins (Georgopoulos and Welch, 1993; Langer and Neupert, 1991). They are classified according to their molecular weights eg. hsp60, hsp70, hsp90. Heat shock proteins are highly conserved and human homologues are found in bacteria and yeasts. Most heat shock proteins are molecular chaperones, a large family of proteins whose functions are to prevent protein

aggregation, promote proper folding of non-native proteins, assist in the assembly of multi-subunit protein complexes, as well as target proteins for membrane translocation (Craig et al., 1994). Hsp60 is a molecular chaperone and deletion of it is lethal as protein assembly essential for normal cellular functions and growth is disrupted (Bukau & Horwich, 1998; (Fayet et al., 1989). Hsp60 binds misfolded proteins and newly synthesised protein intermediates, and facilitates their folding into native conformations. The misfolded proteins are sheltered from the cytosolic environment to prevent aggregation of similarly misfolded proteins into physical masses which would destabilise the cells functionally and physically. Proteins which cannot be refolded into their proper conformation are marked for degradation.

When first isolated most hsp60 was found to be localised to the cytoplasm of prokaryotes and mitochondria in eukaryotes. In mammalian cells, hsp60 has been localised to various cytoplasmic and organelle related locations; including endoplasmic reticulum, peroxisomes, mitochondria (Itoh et al., 1995; Itoh et al., 2002; Soltys & Gupta, 1996). In normal cells hsp60 is assembled into a barrel-shaped hetero-oligomer. The hsp60 units react with proteins and polypeptides which have folded in tertiary formations. Misfolded proteins are ushered into the hsp60 barrel and the lid is provided by another stress protein, hsp10. The properly folded peptides are then released into the cytoplasm. Earlier works have suggested such actions form an important stress response generated within mitochondria, a key player in the initiation of apoptosis through the intrinsic pathway.

The mechanism by which hsp60 associates with the cell membrane is not fully understood. The ability of hsp60 to be incorporated into membrane has been observed

(Torok et al, 1997). The highly conserved C-terminus of hsp60 is involved in membrane targeting. It was found that hsp60 could penetrate efficiently both monolayers and bilayers of phospholipids, and the hsp60 complexes retained ATPase and protein folding activity while bound to the reconstructed membranes. It has also been shown that hsp60 may attach itself to the plasma membrane through protein kinase A-catalysed phosphorylation (Khan et al., 1998). In addition hsp60 may participate in translocation of protein across the cytoplasmic membrane of *E. coli*. Bochkareva et al. (1988) demonstrate that hsp60 maintains newly synthesised pre- β -lactamase in translocation-competent unfolded state for secretion, whilst Kusukawa et al. (1989) report that functionally defective hsp60 compromised such export. An essential role of hsp60 in export of other secretory proteins have also been reported by Lecker et al. (1989) and Battistoni et al. (1995). Translocation across the membrane requires Mg-ATP-promoted discharge of the protein from GroEL.

Functions of hsp60 on the membrane

Heat shock protein 60 translocating to the cell membrane of an apoptotic cell may serve certain functions. There is evidence that hsp60 when incorporated into the cell membrane exists as whole molecules (Ferrarini et al., 1992; Kaur et al., 1993). Surface hsp60 may be involved in processing of proteins for antigenic presentation (Belles et al., 1999; Blander and Horwitz, 1993). This suggests hsp60 may retain its chaperone functions in the membrane of apoptotic cells. It may be chaperoning peptide(s) targeted to the membrane when a cell undergoes apoptosis. These peptides might be secreted in apoptotic cell death, serving as a signal for phagocytes. Alternatively, hsp60 may be keeping certain peptide groups in the membrane properly constituted.

Heat shock protein 60 and immunological response

Heat shock protein 60 (hsp60) has been noted to be released during cellular stress. It has also been identified as an autoantigen especially in patients with autoimmune disease. Elevated levels of anti-hsp60 antibodies have been detected in several autoimmune disorders including juvenile chronic arthritis and diabetes mellitus (de Graeff-Meeder et al., 1993). There have been reports of homology between hsp60 and many known auto-antigens (Jones et al., 1993). It was reported that hsp60 has the capacity to activate macrophages thereby stimulating the innate immune system (Chen et al., 1999; Kol et al., 1999). This observation was followed by reports that hsp60 activates the macrophages through CD14 (Kol et al., 2000), and that the putative receptor for hsp60 is Toll-like receptor-4 complex, Tlr (Ohashi et al., 2000). These studies utilised purified recombinant hsp60 to activate macrophages. Hsp60-mediated activation was abrogated in the presence of blocking antibodies to CD14, and also in Tlr mutant mice. The authors suggest that these findings support an intimate link between tissue hsp60 and inflammation. However hsp60 homologues from *Escherichia coli* and *Mycobacterium tuberculosis* can also activate macrophages in a CD14-independent manner (Lewthwaite et al., 2001).

Reports offering counter evidence have recently emerged. Classically the ligand activating CD14 in conjunction with Tlr has been lipopolysaccharide (LPS), an essential product of the bacterial cell wall. It has been suggested that in earlier studies, the effects attributed to recombinant hsp60 arose from contamination with bacterial LPS. Using polymyxin B-agarose column to deplete LPS and LPS-associated proteins, Gao and Tsan (2003) reported that purified recombinant hsp60 was not able to activate macrophages. It was shown that the LPS-contaminated hsp60 induced production of

tumour necrosis factor-alpha (TNF-alpha) from macrophages even at very low levels. The authors believed that contamination with LPS-associated factors, which are not sensitive to polymyxin B treatment, might also have accounted for the TNF-alpha inducing properties of preparations of recombinant hsp60. A similar finding has been put forward for recombinant hsp70, another stress protein previously shown to activate macrophages (Gao and Tsan, 2003). In the light of these studies further corroborative reports, utilising methods to assess LPS contamination and functional activities of hsp60, will be needed to clarify the interaction of hsp60 and the innate immune system.

Significance of heat shock protein 60 on apoptotic cells

1. Hsp60 may be required for a non-inflammatory response to apoptosis

Our observations on the expression of hsp60 on apoptotic cells are supported by studies using different models. Extending an earlier observation that T lymphocytes infected with HIV-1 virus had increased cytoplasmic levels of hsp60 (Poccia et al., 1996), the authors tested the expression of hsp60 on infected T cells undergoing apoptosis. It was found that the subset of smaller and more granular lymphocytes detected by flow cytometry, probably representing late apoptotic cells, express hsp60 on the membrane (Poccia et al., 1996). It has also been reported that hsp60 probably translocated to the plasma membrane in cardiac myocytes undergoing apoptosis (Kirchhoff et al., 2002; Gupta and Knowlton, 2002). The authors found that total cellular hsp60 was unchanged during hypoxia, but that hsp60 translocated to the plasma membrane fractions in apoptotic cells. These studies also pointed to the association of hsp60 with Bax, a pro-apoptotic member of the bcl-2 family of proteins. It was suggested that when Bax

dissociated from hsp60, it moved to mitochondria to induce release of cytochrome c initiating apoptosis whilst hsp60 moved to the plasma membrane.

It is interesting that hsp60 is expressed on all apoptotic cells which have been examined during the course of this study. Although the potential of hsp60 to stimulate the innate immune system remains to be determined, the non-inflammatory property of apoptotic cells has been the defining nature of apoptosis. In fact uptake of apoptotic cells by macrophages is attended with production of anti-inflammatory cytokines (Fadok et al., 1998; McDonald et al., 1999; Huynh et al., 2002). It appears logical that disposal of apoptotic cells occurring under physiological conditions, eg. development, or during resolution of inflammation is meant to be non-inflammatory. It is probably through defects in recognition and disposal of apoptotic cells that intracellular antigens are presented to the immune system in a stimulatory context. This might be the basis of autoimmune diseases such as systemic lupus erythematosus (SLE) where apoptotic bodies form a distinctive feature of the histological landscape (Potter et al., 2003).

It is very likely that the potential to stimulate immune cells depends very much on the context in which hsp60 is presented to them. Studies reporting the immunostimulatory properties of hsp60 have so far relied on feeding the macrophages free molecules of purified recombinant hsp60 in culture systems. Most of these studies were designed to simulate situations where tissue damage releases cytoplasmic hsp60, such as hypoxia or in infections. It is hypothesised that infections with *Chlamydia* bacteria sensitises the innate immune system to hsp60 and where cardiac myocytes release hsp60, an inflammatory reaction is set up leading to further damage and local inflammation eventually leading to atherogenesis (Kol et al., 1998; Libby et al., 1997; Xu et al.,

1992). However the responses elicited when macrophages encounter hsp60 molecules on the membrane blebs of apoptotic cells would probably be very different from those stimulated when hsp60 is freely released into the circulation in tissue necrosis.

II. Heat shock protein 60 may be used by intracellular bacteria as molecular mimicry

Surface expression of hsp60 is a consistent feature of many intracellular bacteria. Difference in the surface expression of hsp60 has been noted among strains of *Legionella pneumophila*, a gram negative, facultative intracellular bacterium which causes the pulmonary infections called Legionnaire's disease. It infects through the lungs and is taken up by alveolar macrophages (Cianciotto, 2001). This has to do with its ability to subvert the immunological response of macrophages. The virulent strains of *L. pneumophila* have been shown to be expressing higher levels of surface hsp60 (Fernandez et al., 1996). Once inside the host macrophages the synthesis of hsp60 is reduced. Surface hsp60 accounts for the difference in invasiveness of intracellular *Legionella pneumophila* (Garduno, Garduno & Hoffman, 1998). Using HeLa cells as a model for invasion, the authors found that surface hsp60 promoted binding and internalisation of *L. pneumophila* by HeLa cells. The invasiveness of virulent *L. pneumophila* might be suppressed through the antagonistic action of purified hsp60 or substratum-bound hsp60. It had been noted that when taken up by host macrophages bacterial hsp60 is released into mature or newly formed phagosomes, events which correlate with the ability of virulent *L. pneumophila* to abrogate the fusion of phagosome with lysosome (Fernandez et al, 1996). The avirulent strains however do not show this up-regulated expression of surface hsp60.

It had been previously reported that the heat shock proteins hsp70 and hsp90 share a common receptor (Basu et al, 2001). The receptor was identified as the alpha-2M receptor. Hsp60 however does not share this receptor. Certain features of the macrophage receptor for hsp60 have been demonstrated (Habich et al, 2002). The putative receptor is specific for hsp60, and is not antagonised by hsp70 or hsp90. In addition Toll-like receptor (TLR) does not mediate binding of hsp60, as macrophages lacking TLR could bind hsp60. A specific ligand-receptor interaction probably exists for hsp60 and macrophages. Recently hsp60 has been identified as the factor used by the fungus *Histoplasma capsulatum* to target entry into macrophages via phagocytosis (Long et al., 2003). This had been demonstrated in surface staining on flow cytometry and confocal microscopy, as well as immunoblotting. The uptake of *Histoplasma capsulatum* is enhanced by the presence of surface hsp60. As with *L. pneumophila*, the endocytosed *H. capsulatum* is not degraded in the lysosomes. It was however difficult to confirm if the fungal hsp60 was instrumental in preventing phago-lysosomal fusion (Long et al., 2003).

In summary, it appears that phagocytosis of cells or organisms by macrophages may be significantly influenced by surface hsp60 on these entities. Membrane-bound hsp60 appears to target a specific receptor on the macrophages. Recognition by this receptor is linked to phagocytosis, and the degree of uptake appears proportionally related to the level of hsp60 expression on the surface. Intracellular organisms also rely on the hsp60 to prevent fusion of the phagosome and lysosome thereby averting digestive breakdown by the macrophages. The setting in which the membrane hsp60 is presented to the macrophage receptor is likely to be vital to this escape from immune activation, allowing organisms which express surface hsp60 to reside within the macrophages.

The platelet is another entity which possibly uses membrane hsp60 in a similar manner. Platelets undergoing senescence execute a caspase-driven programme. The membrane changes on aged platelets are reminiscent of those on apoptotic cells. Aged platelets express hsp60 on the surface. *In vitro* it had been shown that aged platelets are avidly taken up by macrophages. *In vivo*, during remodelling of a platelet plug covering a defect in the blood vessel, platelets are actively phagocytosed by macrophages (Diegelmann and Evans, 2004). The surface hsp60 on platelets may therefore serve as a membrane marker to facilitate uptake by macrophages.

It may be possible that the manner in which hsp60 is presented by these intracellular organisms is similar to the way hsp60 is displayed on apoptotic bodies. It may be conjectured that displaying hsp60 on the surface serves as an advantage for these microbes. These microbes may be using molecular mimicry. The microbes may be using membrane hsp60 as a disguise for apoptotic bodies. The hsp60 moieties on the membrane would enhance the uptake of these microbes into the macrophages. Subsequent to that important changes emerge. Apoptotic bodies get degraded, whereas intracellular microbes do not. There are probably other factors on the membranes of microbes which prevent degradation. The membrane hsp60 however may serve another important function. The hsp60 moieties carried on the apoptotic bodies may generate a non-inflammatory message for the macrophages, possibly another survival tactic for the microbes.

SUMMARY

Heat shock protein 60 is expressed on the membrane of cells, apparently of all lineages except red cells, dying through apoptosis. This surface expression of hsp60 is an event following the initiation of apoptosis, with a major component of the cytoplasmic pool of hsp60 being translocated to the cell membrane in the process. Hsp60 is also expressed on surface of platelets which are undergoing senescence through the same caspase-driven programme. Intracellular microbes utilise surface hsp60 to gain entry into macrophages. Molecules of hsp60 expressed on the surface may well be a recognition marker for macrophages, with important immuno-regulatory functions.

FUTURE WORK

A major investigation of the role of hsp60 in macrophage clearance of senescent cells would have added considerably to the work if time had permitted. Cell surface hsp60 expression may be a general macrophage recognition mechanism for apoptotic cells. (Mevorach, 2000).

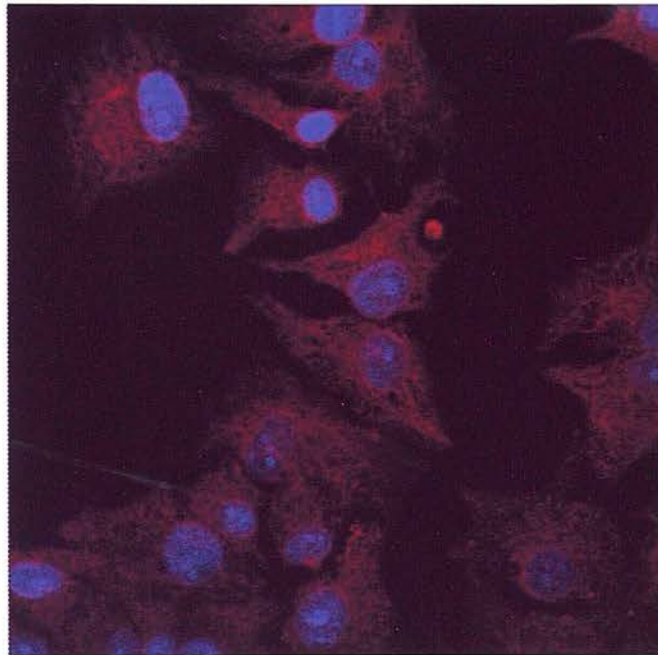
The results obtained from the studies presented in the thesis may be extended in several meaningful directions. The main areas which I would like to develop would be along the following themes, 1) assessing the functional significance of surface hsp60 in phagocytosis, 2) isolating the receptor for hsp60, and 3) tracking the movement of hsp60 in cells during apoptosis using reporter protein.

Functional assessment of the role of surface hsp60 on apoptotic cells may take the form of inhibition studies. Apoptotic THP-1 cells may be co-cultured with untreated THP-1 cells or macrophages, and competitive binding of the putative hsp60 receptor may be effected with saturating the culture medium with recombinant hsp60 protein. In addition competitive binding may also be effected with liposomes bearing purified hsp60. The inhibition of phagocytosis may be compared between hsp60 and phosphatidylserine (PS) on apoptotic cells.

The receptor of hsp60 may be isolated using MEG-01 platelets. Platelets may be aged or induced to undergo apoptosis using calcium ionophore 23827. These may then be fixed (eg. using glutaraldehyde) and presented to macrophages. The reaction mixture will then be lysed, and the proteins differentiated on a SDS-PAGE gel. There may be bands specific to the reaction between the macrophages and the platelets, when compared with control experiments and these bands may be sequenced using MALDI-TOF.

The movement of hsp60 may be tracked using fluorescent reporter proteins eg. GFP (Green Fluorescent Protein), and analysed using time-lapse confocal microscopy. The movement of hsp60 from the time of induction of cell death, translocation from the cytoplasm to the membrane, through to the uptake and engulfment into phagocytes may be followed.

Figure 6.1. Capturing apoptosis. Human hepatoma HUH-7 cells were incubated with 25 μ M monensin for 18 hours. The monolayers of cells were fixed and stained for BOB78. Secondary anti-mouse antibody was TRITC-conjugated (red), with TO-PRO 3 (blue) for nuclear staining. Analysis was effected through confocal microscopy.



Phagocytosis and apoptosis. A human HUH-7 cell was seen developing a phagocytic cup around an apoptotic body marked by its intense staining for BOB78. The distribution of BOB78 within the cell is not different from that within other normal appearing HUH-7 cells. This scenario is reminiscent of cells taking in membrane bound entities under physiological conditions.

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APPENDIX A

MATERIALS FOR CELL BIOLOGY

Cell Culture

Cell lines

THP-1 (human acute monocytic leukaemia; Tshuchiya et al., 1980)

MEG-01 (human megakaryocytic leukaemia; Ogura et al., 1985)

HuH-7 (human hepatoma; Nakabayashi et al., 1982)

MIAPaCa-2 (human pancreatic carcinoma; Yunis, Arimura & Russin, 1977))

Standard culture medium

Dulbecco's Modified Eagle Medium/ Nutrient Mix F12 (1:1), without L-glutamine (DMEM/F12, no. 11880-028 GibcoBrl)

RPMI 1640 Medium
(RPMI, no. 10100-147 GibcoBrl)

10% foetal bovine serum
(no. 14200-059)

2mM L-glutamine
(stock: 100X (200mM) L-glutamine, Gibcobrl)

100 units/ml penicillin + 100µg/ml streptomycin
(stock: 10 000 units of penicillin + 10000µg of streptomycin, GibcoBrl)

Trypsinization

Trypsin-EDTA (0.25% trypsin, 1mM EDTA.4Na) (1X), liquid
(Gibcobrl)

Dulbecco's Phosphate Buffered Saline, 1X, liquid
(Gibcobrl)

Metabolites used in culture

Thymidine (Sigma, product no. T 1895)

Camptothecin (Sigma, product no. C 9911)

Tunicamycin (Sigma, product no. T 7765)

Monensin sodium salt (Sigma, product no. M 5273)

Brefeldin-A (BFA)

γ ,4-dihydroxy-2-[6-hydroxy-1-heptenyl]-4-cyclopentanecrotonic acid λ -lactone
from *Penicillium brefeldianum* (Sigma. product no. B 7651)

Plastic ware

Flasks

Costar 25cm² culture area flasks
(Corning cat no. 3055)

Costar 75cm² culture area flasks
(Corning cat no. 430725)

Plates

Costar 6 well (9.5cm² culture area/well) plate
(Corning cat no. 3506)

12 well (3.8cm² culture area/well) plate
(Corning cat no. 3512)

96 well (0.32cm² culture area/well) plate
(Corning cat no. 3585)

Costar Cell scraper

(Corning cat no. 3011)

APPENDIX B

MATERIALS FOR IMMUNOCHEMISTRY

A. Immunochemistry for flow cytometry

Washes and cell fixative

PBS – 1% BSA (w/v): 1g bovine albumin in 100ml PBS
(Bovine albumin fraction V, Sigma N 8159)

PBS – 1% formalin (v/v)

Fixation

4% paraformaldehyde (w/v)
(prepared from 10% stock with H₂O)

Permeabilisation

100% cold methanol
(stored at -20°C)

Blocking serum

20% normal rabbit serum in PBS
(rabbit serum from Diagnostics Scotland)

Isotype control primary antibody

Mouse IgM negative control (DAKO code no. X 0942)
Antibody specificity for *Aspergillus niger* glucose oxidase, an enzyme neither present nor inducible in mammalian tissues

Secondary antibody

Sheep anti-mouse F(ab')₂ fragment-FITC (fluoro-isothiocyanate)
(Sigma F 2883)

Goat anti-mouse IgM: RPE
(R. Phycoerythrin (RPE)-conjugated goat IgGs (0.2mg/ml) purified from antisera to mouse IgM, Serotec)

Propidium iodide (Sigma product no. P 4170)

PE-conjugated Annexin V
(Cat no. 65875X, BD Pharmingen)

Annexin V binding buffer
10mM Hepes buffer/NaOH (pH 7.4), 140mM NaCl, 2.5mM CaCl_2

Nuclear staining mixture

Nuclear Staining Solution (NSS)
5mg propidium iodide, 0.1mg sodium citrate and 0.3ml Nonidet P40; in 100ml distilled water (protected from light)

RNase A solution
10mg/ml RNase A in 1.12% sodium citrate (diluent)

Flow cytometry instruments and accessories

Immunocytometry

Coulter EPICS XL-MCL Cytometer

Analytical software for Flow Cytometry

Expo™ 32 ADC Analysis for Coulter EPICS XL cytometers
(Beckman Coulter)

B. Immunostaining on slides

Washing Solution

PBS (10x)
1.37M NaCl
27.6mM KCl
81mM Na_2HPO_4
14.7mM KH_2PO_4

Fixation & Permeabilisation

100% Methanol (BDH)

Blocking serum

20% normal rabbit serum in phosphate buffered saline (PBS)
(serum from Diagnostics Scotland)

Primary antibodies

BOB78 (clone 1)

Rabbit anti-human calreticulin IgGs
(Sigma; catalogue no. C4606)

Mouse IgM negative control (DAKO code no. X 0942)
(Antibody specificity for *Aspergillus niger* glucose oxidase, an enzyme neither present nor inducible in mammalian tissues)

Fluorescent secondary antibodies

Polyclonal rabbit anti-mouse immunoglobulins/TRITC
(tetramethyl-rhodamine isothiocyanate R isomer ; DAKO code no. R 0270)

Polyclonal goat anti-mouse immunoglobulins/FITC
(Fluoresceine isothiocyanate isomer 1; DAKO code no. F 479)

Polyclonal swine anti-rabbit immunoglobulins /TRITC
(tetramethyl-rhodamine isothiocyanate R isomer; DAKO code no. R 0156)

Alexa Fluor 546 goat anti-rabbit IgG (H+L) (2 mg/ml)
(Molecular Probes)

Alexa Fluor 546 goat anti-rabbit IgG (H+L) (2 mg/ml)
(Molecular Probes)

Aqueous slide mountant

DAKO

Chamber slides

Lab-Tek II chamber slide with cover (8 well)
(Nalge Nunc International)

Microscopes and camera for fluorescence visualization

Leica DMRB upright microscope (connected to digital camera for imaging)

Leica DC 200 digital camera

Confocal microscopy

Leica DMRE upright microscope with Leica TCS NT confocal system

APPENDIX C

MATERIALS FOR ANTIGEN DETECTION & PURIFICATION

Immunoprecipitation

Protein extraction

TENT buffer

Stock:

20mM Tris, pH 8.0

2mM EDTA

150mM NaCl

1% Triton-X100

Triton-X (Sigma product no. T 8532)

**proteases inhibitors mix added before use*

Antibodies for immunoprecipitation

Primary

BOB78 antibody

Secondary

Rabbit anti-mouse immunoglobulinsIgGs (anti-serum) (3.5mg/ml)

(Affinity-purified rabbit anti-mouse IgM, DAKO code no. Z 0259)

Washing solution ie. extraction buffer without Triton-X

20mM Tris, pH 8.0

2mM EDTA

150mM NaCl

1% Triton-X100

**proteases inhibitors mix added before use*

Protein electrophoresis and Western blotting

RIPA cell lysis buffer

1% Nonidet NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS) in phosphate buffered saline (PBS)

Determination of protein concentration

BCA Protein Assay Reagent kit
(Pierce, catalogue number 23227)

Running buffer

Per 2 Litre solution (5x, pH 8.3)

Glycine	144 g
Tris base (Tris(hydroxymethyl)methylamine)	30g
Sodium doceyl sulphate SDS	10g
ddH2O	2000ml

Transfer buffer

Per 2 Litre solution

Glycine	5.86g
Tris base (Tris(hydroxymethyl)methylamine)	11.625g
Sodium doceyl sulphate SDS	0.75g
Methanol	400ml
ddH2O	1600ml

Washing buffer (TBS)

Per 5 Litre solution (10x, pH 7.4)

Tris base (Tris(hydroxymethyl)methylamine)	121g
Sodium chloride	438.5g
ddH2O	5000ml

Blocking buffer

TBS, 0.1% Tween, 5% Marvel (dried skim milk)

Blotting

Nitrocellulose membrane (Biorad transblot 0.45µm cat. no. 162-0115)

Protein staining on nitrocellulose membrane

Ponceau's solution
(Sigma, product no. B 6008)

Secondary antibody

Polyclonal rabbit anti-mouse immunoglobulins, horseradish peroxidase-linked whole antibodies (DAKO code no. P0260)

Chemiluminescent detection

Enhanced Chemiluminescent detection kit:
ECL Western blotting analysis system
(Amersham Pharmacia Biotech, RPN 2108)

X-Ray film

Kodak X-Omat
(Sigma, catalogue 8532665)

Accessories for Western blotting

Protein assay
Dynaex plate reader

Semi-dry blotting system

Pharmacia LKB Multiphor II electrophoresis unit

APPENDIC C

MATERIALS FOR ELISA

Antibodies for ELISA

Rabbit anti-mouse IgM (specific for μ -chains) (1mg/ml)
(affinity-isolated rabbit anti-mouse IgM, DAKO code no. Z 0457)

Rabbit anti-mouse immunoglobulins, horseradish peroxidase-linked whole
antibodies (DAKO code no. P0260)

Buffers for ELISA

Coating buffer

0.01M phosphate buffer, 0.145M NaCl, pH 7.2

NaH ₂ PO ₄ .H ₂ O	0.345 g
Na ₂ HPO ₄ .12H ₂ O	2.680 g
NaCl	8.474 g

In 1000ml of distilled H₂O, pH 7.2

Washing buffer

0.01M phosphate buffer, 0.5M NaCl, 0.1% Tween 20, pH 7.2

NaH ₂ PO ₄ .H ₂ O	0.345 g
Na ₂ HPO ₄ .12H ₂ O	2.680 g
NaCl	29.220 g
Tween 20 (Sigma cat. no. P7949)	1ml

In 1000ml of distilled H₂O, pH 7.2

0.1M citric acid-phosphate buffer pH 5.0

citric acid. H ₂ O	7.30 g
Na ₂ HPO ₄ .12H ₂ O	23.87 g

In 1000ml of distilled H₂O, pH 5.0

Chromogenic substrate, pH 5.0

OPD tablets (2mg/tablet)	4mg	(Dakoplatts, code no. S2000)
Citric acid buffer	12ml	
30% H ₂ O ₂	5μl	

Chromogenic substrate solution protected from light, stable for 2 hours

1 M Sulphuric acid

55ml 95-97% H₂SO₄
900ml distilled water
Volume adjusted to 1000ml

SURFACE HEAT SHOCK PROTEIN-60 (CHAPERONIN) IS A SPECIFIC MARKER OF APOPTOTIC CELLS

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Introduction

Cells undergoing apoptosis demonstrate distinctive changes in the plasma membrane. Expediency in removal of apoptotic cells has been proposed to be an essential nature of programmed cell death. One of the best characterised is phosphatidylserine (PS), a membrane phospholipid otherwise restricted to the inner leaflet of the plasma membrane. Externalised PS residues have been implicated as key signal for binding and engulfment of apoptotic bodies by phagocytes. Other pro-engulfment participants include CD36, thrombospondin, mer-family receptors, CD32, and CD14. With the myriad of putative factors involved, it remains unclear which play pivotal roles. A monoclonal IgM antibody raised by JA Ross has consistently identified apoptotic cells. It identifies an intracellular antigen (BOB78) which translocates to the surface of plasma membrane when cells undergo apoptosis (Goh et al, ELSO, 2003).

Methods

Visualisation of BOB78 on apoptotic cells has been effected through fluorescent immunocytochemistry. The captured BOB78 antigen was detected through SDS-PAGE and identified through mass spectrometry. Reactivity of BOB78 was verified with purified heat shock protein 60.

Results

The BOB78 antigen is multi-lineage and highly conserved. It is concentrated on the membrane blebs of apoptotic cells. Surface expression of BOB78 is coincident with that of PS, but is lost upon necrotic degeneration. The antigen identified by BOB78 is heat shock protein 60 (chaperonin).

Discussion

We conclude that heat shock protein 60 (chaperonin) is a consistent surface feature of cells undergoing apoptosis. It remains to be studied if hsp60 may be chaperoning molecules which are essential as recognition markers for apoptotic cells.

The following companies have procured the monoclonal antibody BOB78 for commercial purposes based on the findings elaborated in my thesis.

Sigma-Aldrich
3050 Spruce Street
St Louis
MO 63103
e-mail: sigma-aldrich.com

ALEXIS Corporation
Industriestrasse 17
CH-4415 Lausen
Switzerland
e-mail: alexis-ch@alexis-corp.com

HyCult Biotechnology